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Characterization of the Interaction Between R. Conorii and Human Host Vitronectin in Rickettsial Pathogenesis

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CHARACTERIZATION OF THE INTERACTION BETWEEN *R. CONORII* AND HUMAN HOST VITRONECTIN IN RICKETTSIAL PATHOGENESIS

A Dissertation

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“Even if it Breaks Your Heart”

Eli Young Band

Way back on the radio dial,
The fire got lit inside a bright-eyed child
Every note just wrapped around his soul,
From steel guitars to Memphis, all the way to rock and roll

Oh, I can hear ‘em playin’
I can hear the ringin’ of a beat up ol guitar
Oh, I can hear singin’
“Keep on dreamin’ even if it breaks your heart”

Downtown is where I used to wander
Old enough to get there but too young to get inside
So I would stand out on the sidewalk,
Listen to the music playin’ every Friday night

Oh, I can hear ‘em playin’
I can hear the ringin’ of a beat up ol guitar
Oh, I can hear singin’;
“Keep on dreamin’ even if it breaks your heart”

Some dreams stay with you forever,
Drag you around and bring you back to where you were
Some dreams keep on gettin’ better
Gotta keep believin’ if you wanna know for sure

Oh, I can hear ‘em playin’
I can hear the ringin’ of a beat up ol guitar
Oh, I can hear singin’;
“Keep on dreamin’ even if it breaks your heart”

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ABSTRACT

Spotted Fever Group *Rickettsia* are inoculated into the mammalian host during hematophagous arthropod feeding. Once in the bloodstream and during dissemination, the survival of these pathogens is dependent upon their ability to evade innate host defenses until a proper cellular target is reached. The establishment of a successful infection also relies on the ability of the bacteria to attach and invade target cells, as failure to do so results in destruction of the bacterium. *Rickettsia conorii* expresses an outer membrane protein, Adr1, which binds the multifunctional human glycoprotein, vitronectin, to promote resistance to complement mediated killing. Homologs of Adr1 are present in all sequenced pathogenic *Rickettsia* species to date, suggesting that the ability to sequester vitronectin is a conserved attribute for this genus of bacteria. The ultimate goal of my dissertation research was to define the roles of vitronectin acquisition by *R. conorii* in rickettsial pathogenesis. Herein, I characterize the Adr1/vitronectin interaction, demonstrating that this beneficial protein-protein interaction is a heparin-independent, electrostatic interaction that is mediated by the C-terminal region of human vitronectin. By utilizing site-directed mutagenesis, I was able to identify specific amino acids within the Adr1 protein that form interaction with the multimeric form of vitronectin for protection from complement mediated killing. I subsequently employed exogenous vitronectin to examine the role of vitronectin in adherence and invasion of *R. conorii* into phagocytic and endothelial cells. Interestingly, the presence of vitronectin increased the ability of the bacteria to invade monocytes but decreased the ability of the bacteria to invade endothelial cells. In summary, my findings demonstrate a dual role for vitronectin acquisition in rickettsial pathogenesis. This knowledge will be useful to specifically target the *R. conorii*/vitronectin/host cell interaction for the development of potential prophylactic or therapeutic interventions.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 *Rickettsia* and Disease

Bacteria of the genus *Rickettsia* are small (0.3 to 0.5 μm by 0.8 to 1.0 μm), obligate, intracellular, Gram-negative, α-proteobacteria, many of which are clinically relevant human pathogens. (Paddock, 2012; Riley et al., 2012). Mammalian hosts become infected when an arthropod vector, such as a tick, flea or mite takes a blood meal (Azad and Beard, 1998). *Rickettsia* can be found on every continent except Antarctica and are responsible for a number of emerging infectious diseases worldwide (Paddock, 2012). Traditionally, *rickettsia* taxonomy is derived by sequence analysis of various genes including house-keeping genes and immunodominant outer-membrane proteins (Gillespie et al., 2008; Walker and Ismail, 2008). Currently, the bacteria are divided into four groups based on their biological, genetic and antigenic characteristics: the spotted fever group (SFG); the typhus group (TG); the transitional group (TRG); and the ancestral group (AG) (Walker and Ismail, 2008). Members of the SFG, TG and TRG have been associated with human infections and are responsible for a number of significant human diseases including Rocky Mountain Spotted Fever, Mediterranean Spotted Fever and Epidemic Typhus (Azad and Beard, 1998).

Rickettsia are maintained in an enzoonotic cycle between humans, mammals, birds, and hematophagous arthropods (Ceraul, 2012). SFG *Rickettsia* are transmitted by the family of hard ticks known as Ixodidae while TG and TRG *Rickettsia* are transmitted by fleas, lice and mites (Raoult and Roux, 1997). Ticks that carry *rickettsia* can act as both vector and reservoir and distribution of the bacteria is limited to the distribution and seasonality of the tick. (Raoult and Roux, 1997; Azad and Beard, 1998). The tick host can acquire and maintain a rickettsial infection

vertically by transovarial and transstadial transmission, or horizontally, by feeding on a rickettsemic host (Burgdorfer and Brinton, 1975; Walker and Ismail, 2008). After transmission of the bacteria to a tick, the bacteria can infect and multiply in almost all tissues of the tick, including the salivary glands and ovaries (Raoult and Roux, 1997). Infected ticks can then transmit SFG *Rickettsia* to a mammalian host via inoculation of bacteria into the host during a blood meal (McGarry, 2011). The relationship between rickettsiae and tick is not always mutually beneficial. Some species of *rickettsia*, such as *Rickettsia peacockii* are maintained within the tick population as an endosymbiont, while others are detrimental to their tick host's fitness (Harris et al., 2017). Pathological effects in ticks have been described for *R. rickettsii* infection of *Dermacentor andersoni*, *Dermacentor variablis* and *Amblyomma americanum* (Burgdorfer and Brinton, 1975). This suggests that both mammalian and tick reservoirs play a crucial role in maintenance of pathogenic *rickettsia* species in nature.

TG and TRG *Rickettsia* are associated with blood sucking insects and arthropods such as human body lice, fleas and mites. Because these insects are intermittent feeders and are capable of multiple feedings, they have the potential to spread rickettsiae rapidly among susceptible populations causing massive outbreaks of disease (Azad and Beard, 1998). Reservoirs for TG and TRG *Rickettsia* include mammals such as small rodents and humans. Unlike ticks which can serve as both reservoirs and vectors for SFG *Rickettsia* mites act only as vectors for TG and TRG rickettsial species. After uptake of bacteria during a blood meal on a rickettsemic host, the bacteria infect the lining of the gut and are eventually shed in the feces. Transmission of the bacteria to mammalian host occurs by contamination of the bite site with feces shed from the infected insect which can remain infective for years (Raoult and Roux, 1997).

Rickettsia species include some of the most pathogenic bacteria known and as a result they have had a profound impact on human lives over the centuries (Paddock, 2012). The first recorded outbreak of rickettsial infection occurred during the Siege of Naples in 1529 (Walker and Ismail, 2008). The bacterium responsible for this outbreak was later determined to be *R. prowazekii*, the causative agent of epidemic typhus. Throughout the next five centuries, typhus continued to devastate human populations, especially those grouped closely together or displaced by war (Paddock, 2012). During World War I, approximately 30 million people in Russia were afflicted, of which, 3 million perished (Walker and Ismail, 2008). Typhus is also suspected to be a major contributing factor to soldier deaths in the Napoleonic war and the war of Spanish succession from 1710 to 1712 (Paddock, 2012).

RMSF and MSF which are caused by infection with *R. rickettsii* and *R. conorii* respectively, have been described since the beginning of the 20th century (Parola and Raoult, 2006). As reported by Parola et al. (2005), the first clinical appearance of the disease was described by Edward E. Maxey in 1899 (Parola et al., 2005). Then in 1906, Howard T. Ricketts traveled to the Bitterroot Valley in Montana to study a disease known as black measles (Ricketts, 1906a). Ricketts and his assistant demonstrated that black measles, also known as spotted fever, was a tick-borne disease (Ricketts, 1906b; 1907; Gross and Schafer, 2011). The team would go on to study typhus in Mexico where they would isolate the bacterium responsible for illness in the blood of a patient. Unfortunately, this discovery would ultimately lead to Ricketts infection and cause his untimely death. Due to his pioneering work in the field, the shared genera of organisms were later named after him (Wolbach, 1919; Gross and Schafer, 2011).

RMSF and MSF are considered two of the most severe diseases (Lin and Decker, 2012). Infection with the bacterium can cause a wide range of clinical symptoms usually involving fever,

headache, and the characteristic macropapular rash. The rash begins on the extremities, such as the wrist and ankles, and moves toward the trunk of the body indicating systemic infection (Drexler et al., 2016). In MSF, an eschar, defined as a small, non-pruitic lesion surrounded by an erythematous halo where bacteria and immune cells are can be found in approximately 72% of cases present (Paddock et al., 2004; Oteo and Portillo, 2012). If left untreated, infection can lead to increased morbidity and potentially death (Walker and Ismail, 2008). The incubation period from tick bite to initial onset of symptoms occurs between 2 and 14 days with a rash occurring in most patients 3 to 5 days after the onset of fever (Lin and Decker, 2012). When properly diagnosed, treatment with the antibiotic doxycycline, is generally successful with less than a 1% mortality rate; however, if left untreated the fatality rate may be as high as 20 to 25% (Drexler et al., 2016).

R. parkerii, a pathogenic rickettsial species closely related to *R. rickettsii*, has also been shown to cause human illness within the United States (Drexler et al., 2016). Infection with *R. parkerii* can be distinguished from *R. rickettsii* by the presence of an eschar at the site of tick attachment (Paddock et al., 2004; Paddock et al., 2008). TG *Rickettsia* infection is characterized by similar symptomology to RMSF and MSF. Patients experience fever, headache, mental confusion and rash (Walker and Ismail, 2008). If left untreated, typhus can lead to life-threatening complications and eventually death in formerly healthy individuals (Walker and Ismail, 2008).

Rickettsia are obligate, intracellular bacteria and as such can only replicate in the nutrient-rich cytoplasm of the host cell where they have access to the biosynthetic precursors required for growth and survival. The bacteria are capable of infecting cells of many origins, including endothelial cells, monocytes, macrophages and hepatocytes (Walker and Gear, 1985; Walker and Dumler, 1994; Walker, 1997; Walker et al., 1999; Chan et al., 2010; Riley et al., 2010; Curto et

al., 2016). Disease pathogenesis is generally thought to be associated with infection of the endothelium and the subsequent damage to the lining of the blood vessels. This results in generalized vascular inflammation, loss of vascular integrity and increased vascular permeability, termed Rickettsial vasculitis (Walker and Ismail, 2008; Sahni and Rydkina, 2009). Disruption of the endothelial lining leads to increased fluid leakage which causes the distinctive dermal rash (Walker and Ismail, 2008). Widespread damage to the endothelium can lead to severe manifestations of the disease including encephalitis, which leads to delirium, coma and seizures, non-cardiogenic pulmonary edema, interstitial pneumonia, hypovolemic shock, renal failure and occasionally multi-organ failure and disseminated intravascular coagulation (Walker and Ismail, 2008; Sahni and Rydkina, 2009).

In addition, infection of the endothelium results in endothelial cell activation, production of pro-inflammatory cytokines, oxidative stress and inhibition of apoptosis (Walker and Ismail, 2008; Sahni and Rydkina, 2009). Endothelial activation is characterized by functional changes in the cell including increased adhesiveness for platelets, increased expression of intracellular and vascular cell-adhesion molecules (I-CAM and V-CAM), and E-selectin, as well as increased production of plasminogen activator inhibitor-1 (Silverman, 1986; Teysseire et al., 1992; Sporn et al., 1994; Kaplanski et al., 1995; Sporn and Marder, 1996). Infection also induces translocation of nuclear-factor-kappaB (NF-κB) from the cytoplasm to the nucleus of the cell which leads to increased gene expression of pro-inflammatory cytokines and chemokines such as IL-1α, IL-6, IL-8, and chemoattractant protein-1 (Shi et al., 1998; Joshi et al., 2004; Sahni and Rydkina, 2009). This pro-inflammatory response is linked to preservation of mitochondrial integrity, prevention of the caspase cascade and inhibition of apoptosis of *rickettsia* infected cells, therefore promoting bacterial survival and replication (Clifton et al., 1998; Sahni et al., 2013). Endothelial cell infection

also leads to accumulation of intracellular peroxides and superoxide radicals, increased amounts of extracellular H_2O_2, reduction in the levels of cellular thiols and alterations in the activities of important antioxidant enzymes resulting in loss of osmoregulatory control and cell lysis (Sahni and Rydkina, 2009).

Although endothelial cells have been previously considered the main target for rickettsial infection, macrophages, monocytes and hepatocytes have also been identified as major cellular targets (Walker and Gear, 1985; Walker et al., 1994; Walker, 1997; Walker et al., 1999; Cardwell and Martinez, 2009; Chan et al., 2010; Riley et al., 2010). Recent studies involving C3H/HeN mice and Rhesus Macaques models of SFG *Rickettsia* infection demonstrated evidence of bacteria located with the cytoplasm of macrophages and neutrophils both in circulation and in tissues (Banajee et al., 2015; Curto et al., 2016; Riley et al., 2016). This data suggests that interaction of rickettsiae with non-endothelial cells contributes to disease pathogenesis although the mechanisms have yet to be elucidated. To date, there is no commercially available vaccine in place to protect against rickettsial infections. Several potential targets have been recognized, however a universally protective antigen has yet to be identified. Elucidation of the mechanisms of interaction between *rickettsia* and newly idenfitied target host cells could potentially lead to novel and efficacious targets of anti-rickettsial therapies.

1.2 Complement and Complement Proteins

The complement system is the first line of defense against invading pathogenic microbes and is continuously surveying the human body for intruders in a highly efficient manner. It is considered part of the innate immune system and serves as a bridge to adaptive immunity (Walport, 2001a). The system is comprised of more than 30 surface-expressed and soluble proteins, many of which circulate as inactive precursors that require cleavage to become active enzymes (Mathern

and Heeger, 2015). Complement can be activated via three different pathways: classical; lectin and alternative pathways, which converge at the formation of C3 convertase to form the common terminal membrane attack complex (Figure 1.1) (Liszewski and Atkinson, 2015). The classical pathway is activated upon recognition of antigen-bound antibody by the C1 complex. This causes a conformational change in the C1 complex which results in cleavage of C4 and C2 to their respective parts (C4a, C4b, C2a and C2b). C4b then binds to cell surfaces and recruits C2b to form the C4bC2b classical pathway C3 convertase (Mathern and Heeger, 2015). The lectin pathway functions in a similar manner. Mannose binding lectins (MBL) bind to bacterial carbohydrate motifs which activate MBL-associated proteases. These proteases then cleave C4 and C2 eventually yielding C4bC2b MBL C3 convertase on the cell surface (Mathern and Heeger, 2015). Alternative pathway activation occurs spontaneously and continuously at a low rate. The mechanism involves C3 associating with a water molecule to give C3(H20). This complex recruits factor B (fB) and factor D (fD). fD then cleaves fB and C3 generating molecules Bb, C3a and C3b. C3b binds to the surface of a cell and associates with Bb to form C3bBb alternative pathway C3 convertase (Mathern and Heeger, 2015). Upon formation of C3 convertase by the aforementioned pathways, the enzyme cleaves additional C3 molecules forming an amplification loop. This rapidly increases formation of C3 convertase in the blood and in the absence of regulation results in a high concentration of surface deposited C3b that is critical for opsonophagocytosis (Zipfel et al., 2013).

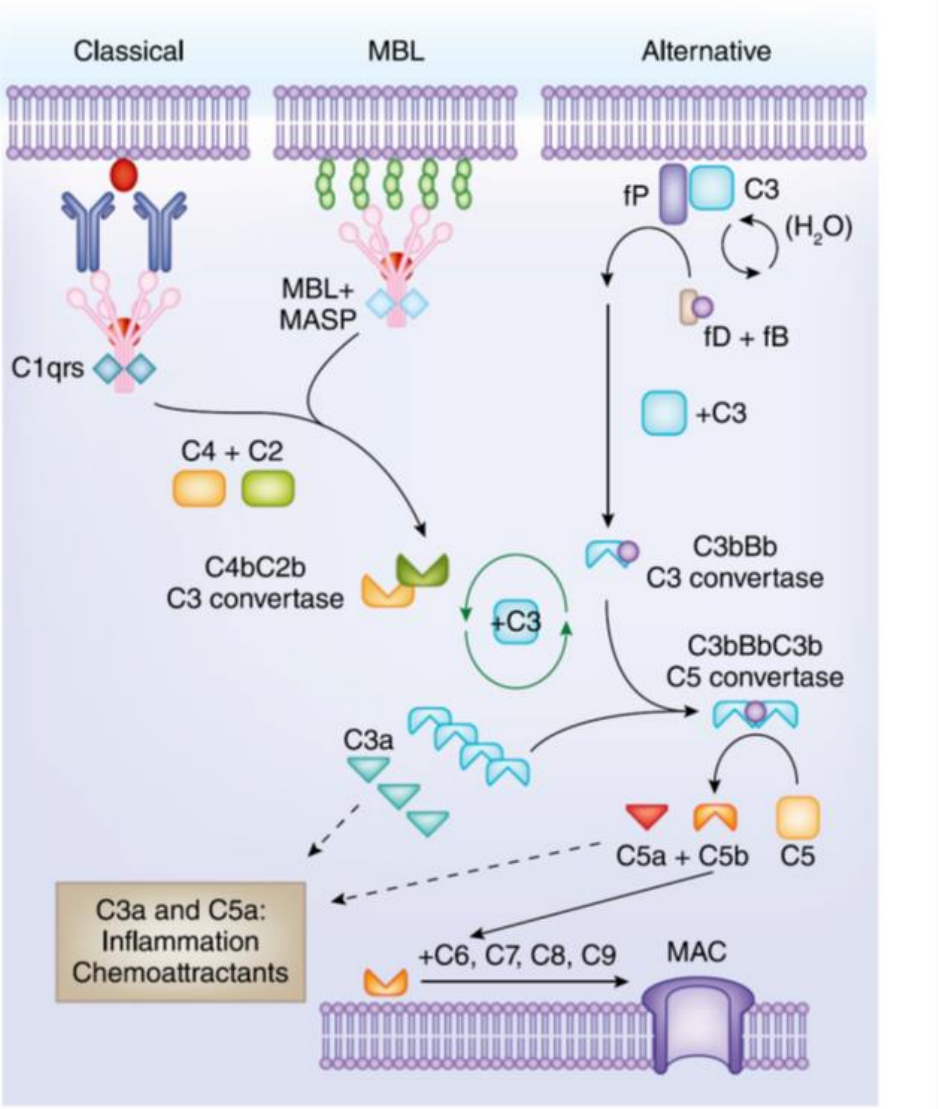

Figure 1.1. Overview of the complement cascade. The complement cascade can be activated via three different mechanisms: the classical pathway, the mannose binding lectin (MLB) pathway or the alternative pathway which converge at the formation of C3 convertease to form the common lytic pathway. The resulting C3 convertase cleaves C3 into C3a and C3b and also forms an amplification loop generating more C3 convertase. The C3 convertases then associated with C3b to form the C5 convertease which cleaves C5 into C5a and C5b. C5b associates with C6, C7, C8 and C9 to form a membrane spanning pore termed the membrane attack complex (MAC). This complex inserts into the cell membrane resulting in cell lysis (Hallstrom and Riesbeck, 2010).

Regardless of the initiating pathway, terminal complement complex formation is initiated when C3 convertase (C4bC2b or C3bBb) binds an additional C3b molecule producing the highly unstable C5 convertase (C4bC2bC3b or C3bBbC3b) (Mathern and Heeger, 2015). This volatile enzyme then cleaves C5 into C5a and C5b, of which C5b proceeds to sequentially bind C6, C7, C8 and multiple C9 molecules to form the membrane attack complex (MAC). The MAC then inserts into the target cell membrane forming a pore which results in cell lysis (Zipfel et al., 2013).

Complement not only functions in direct lysis of target cells, but various other complement cleavage products have effector functions that promote immune system activation and clearance of pathogens. C3a and C5a binds to cells bearing the C3a and C5a receptors, such as neutrophils and macrophages, to activate the cell, induce production of pro-inflammatory cytokines and chemokines and increase chemotactic activity (Zipfel et al., 2013). In addition, C3a also possesses anti-microbial activity against both Gram-negative and Gram-positive bacteria and fungi (Zipfel et al., 2013). Activation of macrophages by complement promotes intracellular killing of engulfed organisms and contributes to T-cell and other antigen-presenting cell activation, expansion and survival (Mathern and Heeger, 2015).

Because the complement cascade has the ability to generate a massive amount of inflammation and cause significant tissue damage, it must be kept under strict control. Activation can be controlled at different steps of the cascade and the importance of these regulators is demonstrated by cases in which deficiency results in disease (Walport, 2001a; b). The complement cascade has both fluid-phase proteins and membrane bound regulators in place that participate in the clearance of complement-coated particles and protect host cells from damage (Meri and Jarva, 2001). The major fluid phase regulatory proteins include C1 inhibitor, C4 binding protein (C4BP), factor H (fH), clusterin and vitronectin (Figure 1.2) (Blom et al., 2009). C1 inhibitor prevents

activation of C1 and subsequent cleavage of C4 and C2, while C4BP accelerates the decay of C4bC2b C3 convertase (Meri and Jarva, 2001; Blom et al., 2009). Although these proteins act via different mechanisms, both inhibit activity of C3 convertase within the classical and lectin pathway therefore limiting activation. Factor H plays a similar role to C4BP by blocking the alternative pathway C3 convertase, however it can block C3 convertase activity via three different mechanisms. fH functions by binding to C3b to inhibit Bb binding, accelerating the decay of C3bBb, and it acts as a cofactor to speed up cleavage of C3b to the inactive iC3b (Meri and Jarva, 2001). Both clusterin and vitronectin act on the terminal complement pathway by binding the C5b-C9 complex which prevents addition of C9 molecules and insertion into the target cell membrane. The resulting protein complexes are soluble and unable to induce cell lysis (Meri and Jarva, 2001). Membrane bound regulators of complement include complement receptor 1 (CR1, CD35), membrane cofactor protein (MCP, CD46), decay-accelerating factor (DAF, CD55) and protectin (CD59) (Meri and Jarva, 2001). CR1, MCP and DAF inhibit C3 and C5 convertases and protectin acts in a manner similar to clusterin and vitronectin by inhibiting insertion of the MAC into the cell membrane (Meri and Jarva, 2001). These regulatory proteins are present are a variety of cell surfaces and serve to protect cells against self-attack.

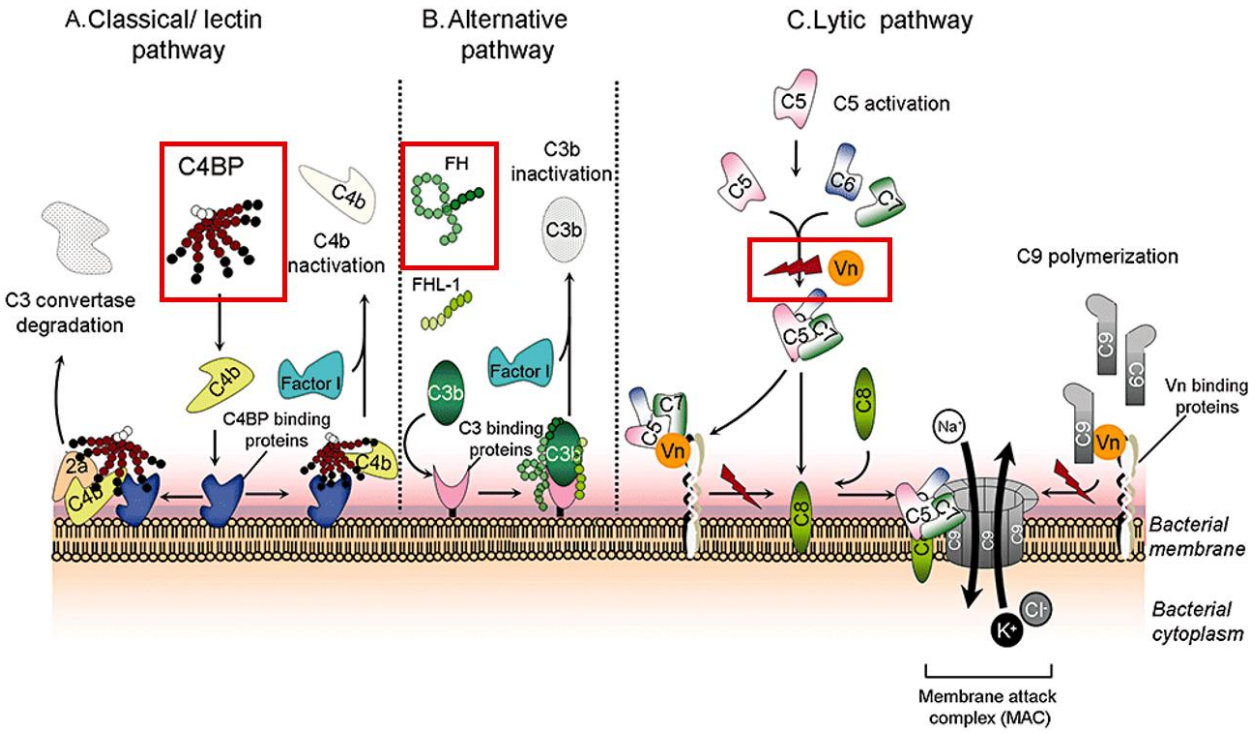

Figure 1.2. Major fluid phase regulatory proteins of the complement cascade. A. C4BP regulates formation of the classical and mannose-binding lectin C3 convertase B. FH controls alternative pathway C3 convertase formation and C. vitronectin regulates formation of the membrane attack complex (Singh et al., 2010b).

Many human pathogens have evolved mechanisms to utilize these host regulatory proteins to their advantage, thereby protecting themselves from complement-mediated attack during the establishment of infection. Both Gram-negative and Gram-positive bacteria such as *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pyogenes* can bind to one or more of the fluid-phase regulators of complement via distinct proteins that are diverse in sequence, but share similar structural integrity (Singh et al., 2010b; Zipfel et al., 2013). Group-A *S. pyogenes* for example, expresses an outer membrane protein termed M protein with an alpha-helical coiled coil structure. (Buffalo et al., 2016). The extracellular portion of this protein is hyper variable and different splice variants are capable of binding several plasma proteins including C4BP and fH to evade

serum killing (Thern et al., 1995; Morfeldt et al., 2001). Interestingly, binding C4BP has also been correlated with resistance to phagocytosis of the bacteria (Horstmann et al., 1988; Carlsson et al., 2003). Another pathogen capable of binding multiple complement regulatory proteins is *Neisseria gonorrhoeae*. This bacterium abundantly expresses an outer membrane protein, Porin, that consists of a 16-stranded transmembrane β-barrel with 8 long surface exposed loops (Massari et al., 2003; Stefanelli et al., 2016). Porin has been demonstrated to be essential for bacterial survival and can bind both C4BP and fH providing the bacteria resistance to all arms of complement mediated attack (Ram et al., 1998; Ram et al., 2001). Furthermore, some human pathogenic yeasts including *Aspergillus* species and *Candida albicans* have also evolved mechanisms to bind complement regulatory proteins thus promoting fungal pathogenesis (Vogl et al., 2008; Lesiak-Markowicz et al., 2011). Clearly, binding plasma proteins is an important mechanism that many different pathogens have evolved to conquer the innate immune defense system of complement, and hence increase survival and pathogenicity.

1.3 *Rickettsia* and Complement

In the process of testing a preventative vaccine, Chan et al (2001) discovered that *R. conorii* are inherently resistant to complement mediated killing in the absence of neutralizing antibodies. This lead to the discovery of two separate mechanisms by which *rickettsia* imitate the natural protected state of host cells where complement is inherently blocked. The first mechanism of resistance identified was by way of factor H binding to the surface of *R. conorii* (Riley et al., 2012). When *R. conorii* was incubated with human serum, Riley et al. (2012) demonstrated a salt-sensitive interaction between fH and the bacterial surface. This interaction inhibited C3 and MAC deposition on the outer membrane and subsequently prevented serum killing (Riley et al., 2012). The rickettsial protein involved in this beneficial protein-protein interaction was identified through

anti-fH immunoprecipitation as the beta-peptide of outer-membrane protein B (OmpB). Additionally, when *R. conorii* was incubated with fH depleted serum, a reduction in serum survival was observed; however, this treatment did not result in the complete clearance of *R. conorii in vitro* suggesting that, although the fH-OmpB interactions play an important role in the inhibition of complement-mediated killing, other mechanisms contribute to the overall serum resistant phenotype (Riley et al., 2012). .

During this time, the Martinez lab was also examining a protein encoded by the *R. conorii* open reading frame which had been previously identified to interact with an unknown mammalian protein and subsequently termed Adr1 (Renesto et al., 2006). Interestingly, the structure of this protein was related to a family of proteins exemplified by *E. coli* OmpX (Figure 1.3) (Riley et al., 2014). Members of this protein family have been documented to acquire host complement regulatory proteins including *Yersinia enterocoliticia* Ail and *Salmonella typhimurium* Rck (Bartra et al., 2008; Ho et al., 2010). Because of the structural similarities, *R. conorii* Adr1 was predicted to be a surface exposed protein that could potentially sequester serum proteins (Riley et al., 2014). Initially, Adr1 expression was verified at the surface of *R. conorii* through flow cytometry utilizing an antibody derived against the predicted extracellular domains of the protein. Upon verification of surface expression, Adr1 was recombinantly expressed on the surface of a serum-sensitive strain of *E. coli* and exposed to human serum. Expression of Adr1 on the surface of the *E. coli* was sufficient to confer serum resistance (Riley et al., 2014). The data suggests that Adr1 is involved in mediated serum resistance in *rickettsia*.

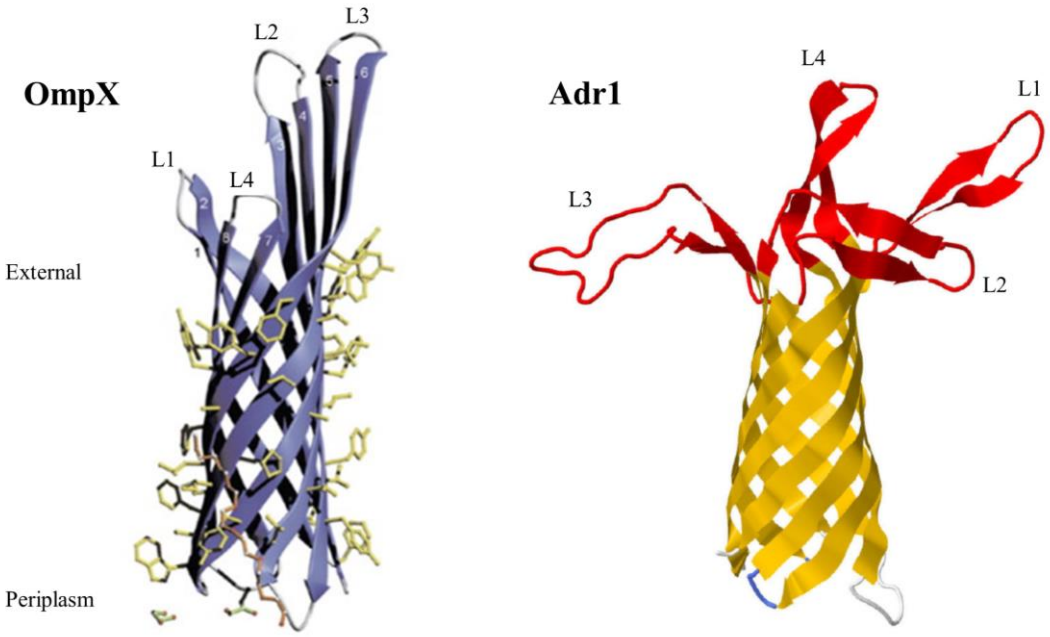

Figure 1.3. Models of the predicted structure of outer-membrane proteins OmpX and Adr1. Pictured here is the predicted structure of *E. coli* OmpX and *R. conorii* Adr1. Both proteins are predicted to consist of 8 transmembrane beta-sheets that form a pore-like structure, 4 periplasmic loops and 4 extracellular peptide loops. Although diverse in sequence, the structural nature of this family of proteins is highly conserved. This image is adapted from (Vogt and Schulz, 1999) and (Riley et al., 2014).

Because depletion of fH from human serum resulted in a partial increase in serum sensitivity, it was hypothesized that *R. conorii* is able to sequester other complement regulatory proteins. To test this theory, the bacteria were incubated with human serum and probed for co-sedimentation with C1 esterase, C4BP, clusterin and vitronectin (unpublished data from the Martinez laboratory). By western immunoblot analysis, vitronectin was demonstrated to interact with *R. conorii* while C1 esterase, C4BP and clusterin did not (unpublished data from the Martinez laboratory). Deposition of vitronectin on the surface of the bacteria was also confirmed by flow cytometric analysis utilizing an anti-vitronectin antibody (Riley et al., 2014). To examine the importance of Adr1-vitronectin interactions, Adr1 was again expressed in a serum-sensitive strain

of *E. coli*, the bacteria were exposed to NHS and binding of vitronectin was evaluated. Western immunoblot and flow cytometric analysis revealed that Adr1 expressing *E. coli* bound significantly more vitronectin then *E. coli* expressing an empty vector (Riley et al., 2014). These findings allude to a correlation between vitronectin acquisition by Adr1 and serum resistance.

A paralog to *R. conorii* Adr1, Adr2, has also been identified as a vitronectin binding protein (Garza et al., 2017). Adr2 is a conserved outer-membrane protein that has been identified in all pathogenic rickettsial species to date and has been implicated in the entry of *R. prowazekii* into non-phagocytic mammalian cells (Gong et al., 2014a; b). In *R. conorii*, Adr2 has been implicated in mediating serum resistance. When the protein was expressed on the surface of a serum sensitive strain of *E. coli* and exposed to human serum, expression of Adr2 was sufficient to mediate serum resistance when compared to *E. coli* expressing an empty vector (Garza et al., 2017). Furthermore, Garza et al (2017) demonstrated that Adr2 binds vitronectin via flow cytometry. Although the group did not demonstrate a role for Adr2 in bacterial adherence to mammalian cells, they discovered a redundant mechanism by which *R. conorii* binds vitronectin to the bacterial cell surface. Taken together the data suggests that binding of fH and vitronectin by *R. conorii* and other rickettsial species including *R. rickettsii*, plays an important role in evasion of the complement cascade and that rickettsial species have evolved multiple mechanisms to evade serum killing while in hematogenous circulation.

1.4 Vitronectin

The open reading frame of vitronectin encodes for 459 amino acids preceded by a 19 amino acid signal sequence (Schvartz et al., 1999). The protein has a predicted molecular mass of 75kD and can be found in circulation as a single chain (75kD) or as two chains linked by a disulfide bond (65kD and 10kD). Post-translational modifications such as sulfatation and phosphorylation

make up about 30% of the total mass; however, no functional significance has been attributed to these modifications to date (Preissner and Seiffert, 1998). The protein consists of various functional domains some of which have specific roles in biological processes while others remain undefined (Figure 1.4). The N-terminal portion consists of a somatomedin-B (SMB) domain that is involved in the binding of plasminogen activator inhibitor-1 (Schvartz et al., 1999). Immediately following the SMB domain is a cell receptor binding site characterized by an arginine-glycine-aspartic acid (RGD) sequence. This domain mediates attachment of cells to the extracellular matrix though specific integrin receptors (Schvartz et al., 1999; Lossner et al., 2009). The protein also contains 4 hemopexin-like domains that are putative heme-binding motifs although a specific function is still unclear (Singh et al., 2010b). Additionally, vitronectin has three heparin binding domains spanning resides 82-137, 175-219 and 448-361 and a C-terminal region with an unknown function (Liang et al., 1997b). Interestingly, many bacterial pathogens have evolved mechanisms to sequester vitronectin and the majority bind within this C-terminal region (Hallstrom et al., 2016).

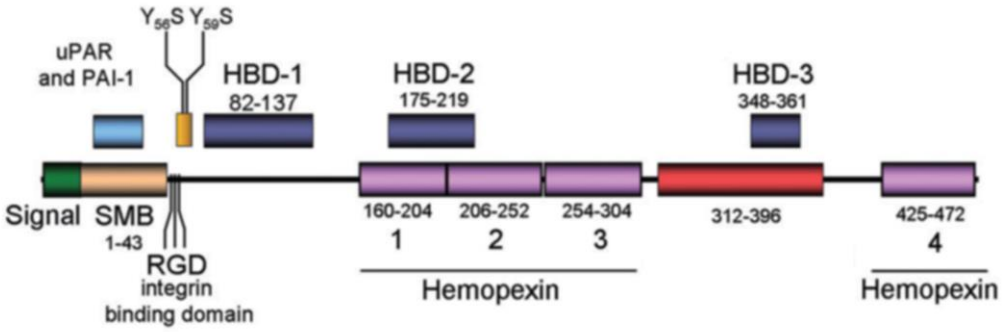

Figure 1.4. Schematic representation of the various domains found in vitronectin. Pictured here are the various functional domains of vitronectin. The N-terminal somatomedian domain depicted in tan binds plasminogen activator inhibitor-1 which is followed by the RGD domain that interacts with cellular integrin receptors. The 4 hemopexin domains that are predicted heme binding motifs are shown in purple. The 3 heparin binding domains are represented here in blue and the C-terminal region with an unknown function is depicted in red (Singh et al., 2010b).

Vitronectin was first identified in 1967 as an inhibitor of the membrane attack complex and subsequently named S-protein for serum spreading factor protein, but was later renamed due to its adhesive properties (Hayman et al., 1983; Schvartz et al., 1999). It is a multifunctional human glycoprotein that is synthesized in the liver and secreted into the plasma (Preissner and Seiffert, 1998). This protein is an important part of the extracellular matrix and as such plays a critical role in many biological processes including cell migration, tissue repair, adhesion, angiogenesis and regulation of membrane attack complex formation (Preissner and Seiffert, 1998; Singh et al., 2010b). It is found at a high concentration in plasma (between 200 to 700 μg/ml) and within many human tissues. Concentrations are particularly high in the liver, tonsils, heart, skeletal muscle, duodenum and lungs (Singh et al., 2010b). Additionally, vitronectin is found at high concentrations in some malignant carcinomas suggesting that it may play a role in tumor development (Berglund et al., 2008). The protein exists in both a monomeric and multimeric form. Most of the circulating protein in the blood exists as a monomer while extravascular vitronectin is usually found in a cell bound multimeric form (Peterson, 1998).

Vitronectin-mediated serum resistance is well-documented for many bacterial pathogens. Both Gram-negative and Gram-positive pathogens including *Helicobacter pylori*, *Moraxella catarrhalis*, *Neisseria meningitidis*, *Streptococcus pneumoniae, Yersinia pestis* and *Pseudomonas aeruginosa* can obtain vitronectin by one or more outer membrane proteins to evade complement-mediated killing (Sa et al., 2010; Singh et al., 2010a; Griffiths et al., 2011; Bartra et al., 2015; Hallstrom et al., 2015; Kohler et al., 2015; Richter et al., 2016). For example, both non-typeable and typeable strains of the respiratory pathogen *Haemophilus influenzae*, can acquire vitronectin by several different proteins: Protein E, Protein F, Protein H and *Haemophilus* surface fibrils (Hsf) (Hallstrom et al., 2006; Hallstrom et al., 2009; Singh et al., 2011; Su et al., 2013; Al-Jubair et al.,

2015) This redundancy ensures vitronectin acquisition and protection from the MAC. The urogenital pathogen, *Haemophilus ducreyi*, an outer-membrane protein termed ducreyi serum resistance protein (DsrA), was demonstrated to bind to vitronectin (Elkins et al., 2000). When *H. ducreyi* DsrA knockout mutants were utilized to infect human volunteers, the patients did not develop an infection. This suggests that vitronectin mediated serum resistance contributes to the infectivity of this bacteria (Bong et al., 2001)

Bacteria can also sequester vitronectin to facilitate adherence to and invasion of target host cells. Distinct binding sites for bacteria and integrins exist within the functional domains of vitronectin and as such bacteria can utilize this to their advantage (Singh et al., 2010b). The respiratory pathogen, *S. pneumonia*, preferentially binds multimeric vitronectin to enhance adherence to respiratory epithelial cells. The bacteria could also bind monomeric vitronectin, but to a lesser extent indicating that cell-bound vitronectin was the preferable target for adhesion (Bergmann et al., 2009). Binding of vitronectin by *Pseudomonas aeruginosa* increases adherence and internalization into A549 alveolar epithelial cells. When antibodies against vitronectin and $\alpha_v\beta_5$ were utilized, bacterial binding and invasion was significantly decreased demonstrating a direct role for a vitronectin mediated adherence and invasion (Chhatwal et al., 1987; Leroy-Dudal et al., 2004). Vitronectin mediated adherence has also been demonstrated for other pathogens, such as *Streptococcus pyogenes*, *Enterococcus faecalis*, *Neisseria meningitidis* and the dental bacteria, *Porphyromonas gingivalis*; however, whether or not this interaction also plays a role in mediated serum resistance remains to be elucidated (Liang et al., 1997a; Olczak et al., 2001; Tyriak and Ljungh, 2003; Sa et al., 2010; Singh et al., 2010b).

Vitronectin acquisition is an advantageous characteristic shared by many human bacterial pathogens. These bacteria have co-evolved mechanisms to sequester vitronectin to evade serum

killing and facilitate adherence to and invasion of host cells. We have previously demonstrated that *R. conorii* expresses a conserved outer-membrane protein, Adr1, that binds to vitronectin to elude complement mediated killing. Therefore, we theorize that like other blood-borne bacterial pathogens, *Rickettsia* species can acquire vitronectin to evade complement mediated killing and to facilitate interactions with target host cells. In this body of work, we will further explore this hypothesis by elucidating the biochemical characteristics of the *R.conorii* Adr1/vitronectin interaction and examining how acquisition of vitronectin contributes to the establishment of a successful infection. Understanding the molecular mechanisms by which pathogenic *Rickettsia* species establish infections in mammalian hosts will hopefully better guide us into developing novel therapeutics, vaccine candidates and a greater understanding of rickettsial pathogenesis.

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CHAPTER 2
THE *RICKETTSIA CONORII* ADR1 INTERACTS WITH THE C-TERMINUS OF HUMAN VITRONECTIN IN A SALT-SENSITIVE MANNER

2.1 Introduction

Spotted Fever group (SFG) *Rickettsia* are Gram-negative, obligate intracellular bacteria that are transmitted to a mammalian host when an arthropod vector takes a blood meal (Riley et al., 2012). Members of the SFG include the human pathogens *R. conorii* and *R. rickettsii*, the etiologic agents of Mediterranean Spotted Fever and Rocky Mountain Spotted Fever, respectively. Upon inoculation into a host, the bacteria can spread throughout the body via the bloodstream and parasitize cells of many origins, including endothelial cells, monocytes, macrophages and hepatocytes (Walker and Gear, 1985; Feng et al., 1994; Walker, 1997; Walker et al., 1999; Riley et al., 2016). Infection of endothelial cells can lead to disruption of the endothelial lining and increased fluid leakage which causes the characteristic macropapular rash, thus the name Spotted Fever (Walker and Ismail, 2008). If left untreated, infections can lead to severe manifestations of the disease such as renal failure, non-cardiogenic pulmonary edema, interstitial pneumonia and ultimately death (Walker and Ismail, 2008). When rickettsial infection is properly diagnosed, treatment is generally successful; however, misdiagnosis is common due to initial non-descript flu-like symptoms, which leads to increased morbidity and mortality (Chan et al., 2009; Riley et al., 2014).

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Because of the obligate intracellular nature of SFG *Rickettsia*, the bacteria must bind to and invade a host cell in order to survive and proliferate (Walker and Ismail, 2008). However, during arthropod feeding and *rickettsia* dissemination, the bacteria are extracellular and as a result are exposed to the hostile environment of the mammalian bloodstream (Riley et al., 2014). While outside the safety of the host cell cytosol, the bacteria are exposed to the bactericidal effects of the host's complement system and survival of these bacteria are dependent upon their ability to evade killing until a proper cellular host is reached (Riley et al., 2012).

The complement system contributes to both the innate and adaptive immune system and serves as the first line of defense against invading organisms. Complement is composed of fluid-phase and membrane bound proteins that can be activated through three different mechanisms (Singh et al., 2010a). The classical, the lectin and the alternative pathways are initiated by antibodies or various proteins that recognize structures on the surface of a microbe and the pathways converge at the formation of C3 convertase to form the common lytic pathway (Blom et al., 2009). This results in the deposition of membrane attack complex (MAC) proteins C5b through C9 on the surface of the pathogen and accumulation of these proteins leads to formation of a lytic pore in the membrane which causes osmotic cell lysis (Singh et al., 2010b). Other functions of complement include increased opsonization of the pathogen by binding of C3b components to the surface of the microbe and stimulation of inflammatory responses with the proteins C3a, and C5a (Blom et al., 2009).

The complement cascade must be kept under strict control, bercause activation can result in significant inflammation and can attack both foreign molecules as well as a self components. As such, the host utilizes a series of regulatory proteins which include Factor I, Factor H, C4-binding protein, vitronectin and clusterin (Singh et al., 2010b). These proteins associate with the

surface of host cells in order to control and block complement activation (Hallstrom et al., 2015). Many bacterial pathogens, including *Moraxella catarrhalis, Neisseria meningitidis, Streptococcus pyogenes, S. pneumoniae, Staphylococcus aureus,* and *Pseudomonas aeruginosa* have evolved mechanisms to utilize these host regulatory proteins to their advantage, thereby protecting themselves from complement-mediated attacks (Liang et al., 1997; Singh et al., 2010a; Griffiths et al., 2011; Voss et al., 2013; Riley et al., 2014; Hallstrom et al., 2015).

A previous report demonstrated that *R. conorii* is inherently resistant to complement-mediated killing when exposed to human serum (Chan et al., 2011). This information led to the discovery of a protein that is expressed on the surface of *R. conorii*, termed Adr1, that contributes to the serum resistance phenotype by binding the multifunctional human glycoprotein vitronectin (Riley et al., 2014). Vitronectin binds to complement proteins C5b-C7 and C9 to inhibit deposition of the MAC on the bacterial surface (Singh et al., 2011). The *R. conorii* vitronectin-binding protein, Adr1, is a conserved outer membrane protein that is predicted to contain 8 trans-membrane beta sheets that form a membrane spanning barrel, as well as four connecting beta strands termed "loops" that protrude into the extracellular environment (Vogt and Schulz, 1999; Riley et al., 2014). Our lab previously demonstrated that two of these domains, loops 3 and 4, were sufficient to interact with vitronectin and thus, mediate resistance to serum killing when Adr1 proteins containing either loop 3 or loop 4 were expressed in a serum-sensitive strain of *E. coli* (Riley et al., 2014). Interestingly, homologs of Adr1 are present in every sequenced rickettsial species to date and the deduced amino acid sequences of loop 3 and loop 4 are almost 100% conserved among spotted fever group rickettsial species (Riley et al., 2014), suggesting that resistance to serum mediated killing mediated by Adr1 may be a widespread virulence attribute in this class of obligate intracellular pathogens.

In the present study, we further analyzed the interaction between Adr1 and vitronectin with the intention of understanding in detail the mechanisms of interaction. We demonstrated that the interaction of Adr1 with vitronectin is sensitive to increasing salt concentrations, and not competitively inhibited by increasing concentrations of heparin. Using various truncated, recombinant vitronectin peptides, we also demonstrate that the Adr1-vitronectin interaction maps to a region located in the C-terminal domain of vitronectin. Furthermore, we utilized site-directed mutagenesis to determine the specific amino acids located within loop 3 and 4 of Adr1 that are critical in mediating resistance to complement-mediated killing in serum.

2.2 Materials and Methods

2.2.1 Construction of Mutants in Adr1 by PCR

Plasmids pJP01-L3 and pJP01-L4 as previously described (Riley et al., 2014) were utilized as a template for quick-change site-directed mutagenesis PCR to mutate individual lysine residues in each loop to alanine residues. Briefly, plasmids pJP01-L3 and pJP01-L4 contain the gene for an Adr1 derivative with only intact loop 3 or loop 4, respectively. All other loops have been reduced to the bare minimum amino acids necessary to maintain structural integrity of the protein (Riley et al., 2014). Primers for mutations can be found in appendix A located at the end of chapter 4. In each case, parental DNA was digested using DpnI leaving mutant plasmid, which was then transformed into MaxEfficiency DH5-alpha-T1 (Life Technologies) and sequenced. Mutants were constructed for single amino acid substitutions as well as multiple and multiple, sequential amino acid substitutions for all 6 lysine residues in pJP01-L3 and pJP01-L4.

2.2.2 Bacterial Strains and Culture Conditions

E. coli BL21 (DE3) (pJP01, pJP01-L3, pJP01-L4, pET22b, pJP01-L3 mutants and pJP01-L4 mutants) were cultured in Luria-Bertani (LB) broth at 37°C with 50 μg/mL of carbenicillin

overnight. Bacteria were then diluted 1:10 into fresh media and grown to an OD_{600} between 0.5 and 1.0. Protein expression was induced with 0.6 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and cultures were grown for 4 h at 37°C.

2.2.3 Antibodies

Rabbit Anti-Adr1 peptide polyclonal antibodies (pAb) were utilized for immunoblot analysis of Adr1-expressing *E. coli* as previously described (Riley et al., 2014). Anti-*E. coli* RNA polymerase monoclonal IgG3 antibody (Ab) was purchased from Affinity Bioreagents. Rabbit IgG anti-human vitronectin pAb was purchased from Complement Technology and sheep anti-human vitronectin IgG was from AbD Serotec. Mouse IgG anti-Histidine tag pAb directly conjugated to horseradish peroxidase (HRP) was purchased from GenScript. Both donkey anti-mouse IRDye 680 IgG and donkey anti-rabbit IRDye 800 IgG were purchased from LiCOR biosciences. HRP-conjugated goat anti-rabbit IgG was from Sigma and donkey anti-sheep IgG was purchased from Thermo-Fisher.

2.2.4 Vitronectin Binding Assay

To evaluate vitronectin binding, *E. coli* BL21 (DE3) containing plasmids pJP01, pET22b, or mutants were grown as described previously. Approximately $1x10^6$ colony forming units (cfu) of each construct was washed with PBS and resuspended in 200μL PBS containing 25% normal human serum (NHS) pooled from 5 healthy individuals (Innovative Research). For analysis of vitronectin binding in the presence of NaCl or heparin, NaCl was purchased from Fisher Chemical and Heparin Sodium Salt was purchased from MP Biomedicals, LLC. The Adr1 and mutant expressing bacteria were resuspended in 100μL PBS containing increasing concentrations of either NaCl (0M, 0.25M, 0.50 M or 1.0M) or heparin (0nM, 1nM, 2nM, 4nM, 8nM, 12nM, 16nM, 20nM, or 500nM). 100μL of 50% NHS was then added to the heparin/*E.coli* or NaCl/*E.coli* solution for

final NHS concentration of 25%. For analysis of binding of vitronectin peptides, $1x10^6$ cfu Adr1 expressing *E. coli* were resuspended in a 100μL of a 5μM solution of each indicated purified recombinant peptide that had been expressed and isolated from HEK293T cells (Singh et al., 2010a). For analysis of vitronectin binding to *R. conorii*, a live, frozen stock of *R. conorii* was allowed to come to room temperature and resuspended in 50μL of a heparin or salt solution at concentrations previously mentioned and 50μL of 50% NHS was added for a final NHS concentration of 25%. To assess differences in multimeric and monomeric vitronectin binding to mutant expressing *E. coli*, multimeric and monomeric vitronectin were purchased from Innovative Research. Roughly $1x10^6$ cfu of Adr1 and mutant expressing bacteria were washed and resuspended in 25μL of PBS. 25μL of 50ng/mL of monomeric or multimeric vitronectin was then added for a final concentration of 25ng/mL vitronectin. Adr1 expressing *E. coli* were incubated on ice when bacteria were resuspended in NHS whereas bacteria resuspended in monomeric, multimeric or recombinant peptide vitronectin were incubated at room temperature. *R. conorii* were also incubated at room temperature. All samples were allowed to bind for 1 h with gentle agitation, bacteria were washed 3 times in PBS and vitronectin binding was determined by SDS-PAGE and western immunoblotting using rabbit anti-human vitronectin or sheep anti-human vitronectin as primary antibody and goat anti-rabbit IgG HRP or donkey anti-sheep IgG HRP as secondary antibody.

2.2.5 Serum Resistance Assay

Normal human serum pooled from 5 healthy individuals (NHS) (Innovative Research) was stored at -80°C as aliquots until use. *E. coli* BL21 (DE3) containing pJP01-L3, pJP01-L4, pET22b or loop 3 and loop 4 mutants were grown as described above. Approximately 1 x 10^6 bacteria were then washed with PBS and resuspended in either 200 μL of PBS or 200 μL PBS containing

10% NHS. Bacteria were incubated at 37°C for 15 min with agitation and then serially diluted in PBS and plated on LB-Agar plates. After overnight incubation, colony-forming units (cfu) were evaluated. Experiments were performed in triplicate with a minimum of 3 replicates for each experiment. Data is presented as the cfu of bacteria in PBS/cfu of bacteria in 10% NHS after 15 min of incubation and plotted on a logarithmic scale. Expression of Adr1or Adr1 mutant proteins was verified by western immunoblotting using rabbit anti-Adr1 or mouse anti-histidine tag antibodies as described above.

2.2.6 Bacterial Fractionation

Induced *E. coli* BL21 (DE3) cultures (250 mL) were pelleted, resuspended in 5 mL PBS containing 1x protease inhibitor cocktail and then fractionated to enrich for outer-membrane proteins as described (Hancock and Nikaido, 1978). Briefly, bacteria were twice lysed in a French Pressure cell (1,500 psi) and unbroken cells were cleared by centrifugation at 3,000 g for 15 min at 4°C. Sarkosyl was added to the resulting supernatant (total cell lysate) to a final concentration of 0.5% and incubated with rotation at room temperature for 10 min to extract inner membrane proteins. Outer membrane proteins were pelleted by ultracentrifugation for 1 h at 100,000 g and resuspended in 2x sample buffer. Total cell lysate, inner membrane fraction and outer membrane fraction were resolved by SDS-PAGE and stained with Coomassie blue for visualization of total protein content and analyzed by western immunoblotting with anti-Adr1 antibodies.

2.2.7 Statistical Analysis

Statistical Analysis was performed using a one-way ANOVA with a Neuman-Keuls post hoc test or a Kruskal-Walis one-way ANOVA test to compare differences between more than two groups as indicated. Differences were considered significant with a *p*-value less than or equal to 0.05 using Graph-Pad Prism version 5.0b (GraphPad Software).

2.3 Results

2.3.1 Vitronectin binding is significantly reduced by increasing concentrations of NaCl, but is not substantially inhibited by heparin

Vitronectin contains many different functional regions that mediate its various roles in the human host (Singh et al., 2010a). Heparin-binding domains have been demonstrated to be involved in the interaction between vitronectin and Outer-membrane protein C (Opc) of *N. meningitis*, Ubiquitous surface protein A2 (UspA2) of *M. catarrhalis*, Lpd of *P. aeruginosa* and several other pathogenic bacteria (Sa et al., 2010; Griffiths et al., 2011; Hallstrom et al., 2015). The charge of the individual amino acid residues within the binding domains of the outer membrane proteins of *S. pneumoniae* and *Haemophilus influenzae* have also been documented to play a role in binding (Hallstrom et al., 2009; Voss et al., 2013). Because of the high concentration of positively charged lysine residues located within loops 3 and 4 of Adr1, we hypothesized that these residues would play an important role in the protein-protein interaction. To determine if the *R. conorii* Adr1/human vitronectin interaction is a result of Adr1 interacting with the heparin binding domains or is an electrostatic interaction, Adr1 was expressed at the surface of *E. coli* and exposed to human serum in the presence of either increasing concentrations of heparin or NaCl. As the concentration of heparin in soluation was increased 0 nM to 500 nM, which is approximately 3 times the concentration found in human plasma (Engelberg, 1961), there was no significant difference in ability of vitronectin to bind to Adr1 (Figure 2.1A). In contrast, increasing concentrations of NaCl in solution ranging from 0 M to 1 M in the reaction, competitively inhibited the interaction between vitronectin and Adr1 (Figure 2.1B). Interestingly, when increasing concentrations of both NaCl or heparin were added to intact *R. conorii* cells in the presence of NHS, there was no significant difference in the ability of vitronectin to bind to *R. conorii* (Figures 2.1 C and 2.1D). These data suggest that *R. conorii* and other pathogenic rickettsial species may

possess additional factors that are sufficient to interact with vitronectin and may contribute to this phenotype. Nevertheless, this data suggests that the heparin-binding domains of vitronectin are not involved in the Adr1/vitronectin interaction, and that specific charged amino acid residues in loop 3 and 4 are likely critical in mediating this protein-protein interaction.

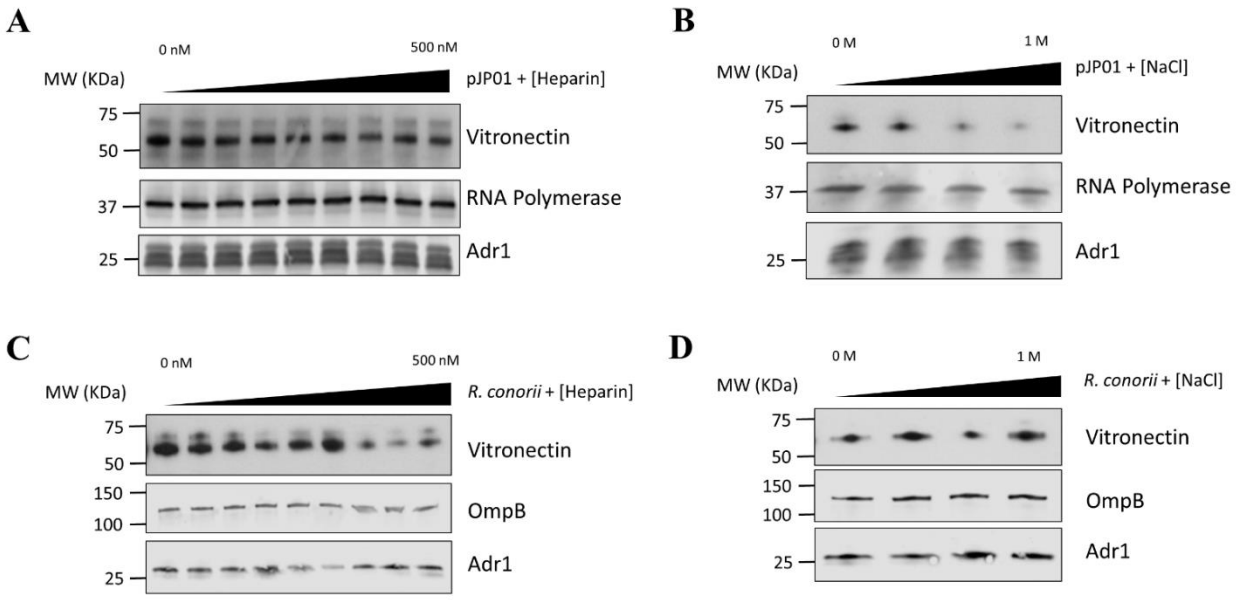

Figure 2.1. Vitronectin binding to *E. coli* expressing Adr1 or *R. conorii*. A & B. Western immunoblot analysis of vitronectin binding to *E. coli* expressing Adr1 in the presence of increasing concentrations of heparin (0nM to 500nM) or NaCl (0M to 1M). Equal loading in each experiment was verified using an *E. coli* RNA polymerase antibody. Verification of Adr1 expression was validated using anti-Adr1. Data are representative of at least 3 independent experiments. C & D. Western immunoblot analysis of vitronectin binding to *R. conorii* in the presence of increasing concentrations of heparin or NaCl. Equal loading in each experiment was verified using anti-OmpB and anti-Adr1 antibodies. Data are representative of 2 replicates.

2.3.2 Adr1 binds within the C-terminal region of vitronectin between amino acids 363 and 373

We next wanted to identify the region(s) within vitronectin that mediate binding to Adr1. The C-terminal region (amino acids 312-396 depicted in Figure 2.2A) of vitronectin is well documented to mediate binding of serum resistance proteins from bacterial pathogens such as Protein E from *H. influenzae*, UspA2 from *M. catarrhalis*, and the PspC protein from *S. pneumoniae* (Singh et al., 2010a; Griffiths et al., 2011; Singh et al., 2011; Voss et al., 2013; Hallstrom et al., 2016). Because this region plays an important role in many interactions of both Gram-negative and Gram-positive bacteria, we hypothesized that Adr1 would also bind within the C-terminal region. To test this hypothesis, we performed vitronectin binding assays using a series of recombinant, truncated vitronectin peptides demonstrated in a silver stain in Figure 2.2B. (Singh et al., 2010a; Su et al., 2013)) and Adr1-expressing *E. coli* BL21(DE3). As shown in Figure 2.2C, peptides Vn 80-396, Vn 80-379, Vn 80-373 and Vn Δ352-362 were sufficient to bind to Adr1 expressed at the outer membrane of *E. coli*. Our data demonstrates that the region between amino acid 363 and 373 within the C-terminal region of vitronectin is involved in the binding of Adr1 and further demonstrate that Adr1-vitronectin interactions do not require heparin-binding domains.

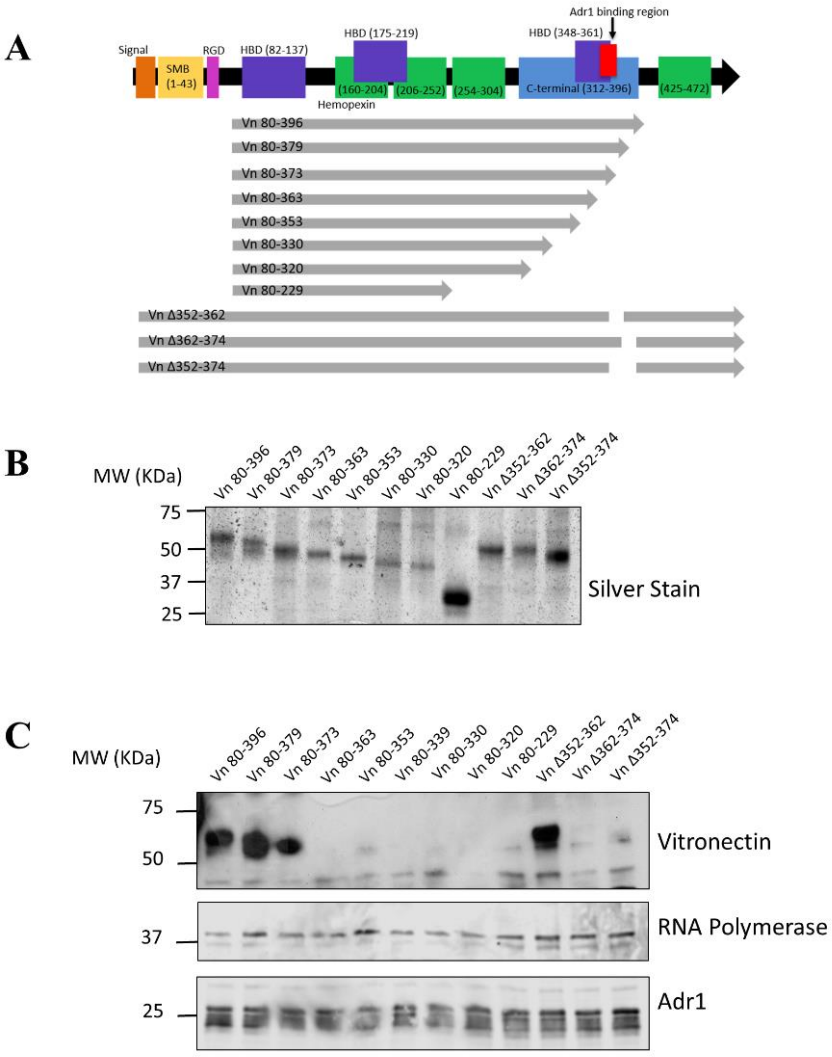

Figure 2.2. Functional domains in vitronectin and peptides constructed for analysis of Adr1 binding region. A. Full length vitronectin with noted functional domains is depicted in the top black arrow with the constructed peptides depicted in gray arrows below. B. Silverstain of vitronectin peptides utilized. The first 8 peptides begin at amino acid 80 and are progressively truncated at the C-terminus. The 3 full length peptides contain a deletion within the C-terminal region. C. *E. coli* expressing Adr1 binds to the 3 longest vitronectin peptides and the full-length peptide with a deletion at amino acids 352-362. Equal loading is demonstrated with anti-E. coli RNA polymerase and verification of expression of Adr1 is verified using anti-Adr1. Data are representative of at least 3 replicates.

2.3.3 Serum survival of *E. coli* expressing Adr1 lysine to alanine mutants

We have previously demonstrated that extracellular Adr1 loops 3 and 4 are sufficient to both mediate resistance to complement-mediated killing and bind vitronectin (Riley et al., 2014).

We, therefore, sought to identify residues within each loop that were responsible for the interaction. We utilized the previously constructed plasmids, pJP01-L3 and pJP01-L4 (Riley et al., 2014) which encode Adr1 proteins expressing only loops 3 or 4, respectively as templates for Site-directed mutagenesis to substitute positively charged lysine residues to uncharged, non-polar alanine residues. A summary of the constructs used in these experiments is depicted in Figure 2.3.

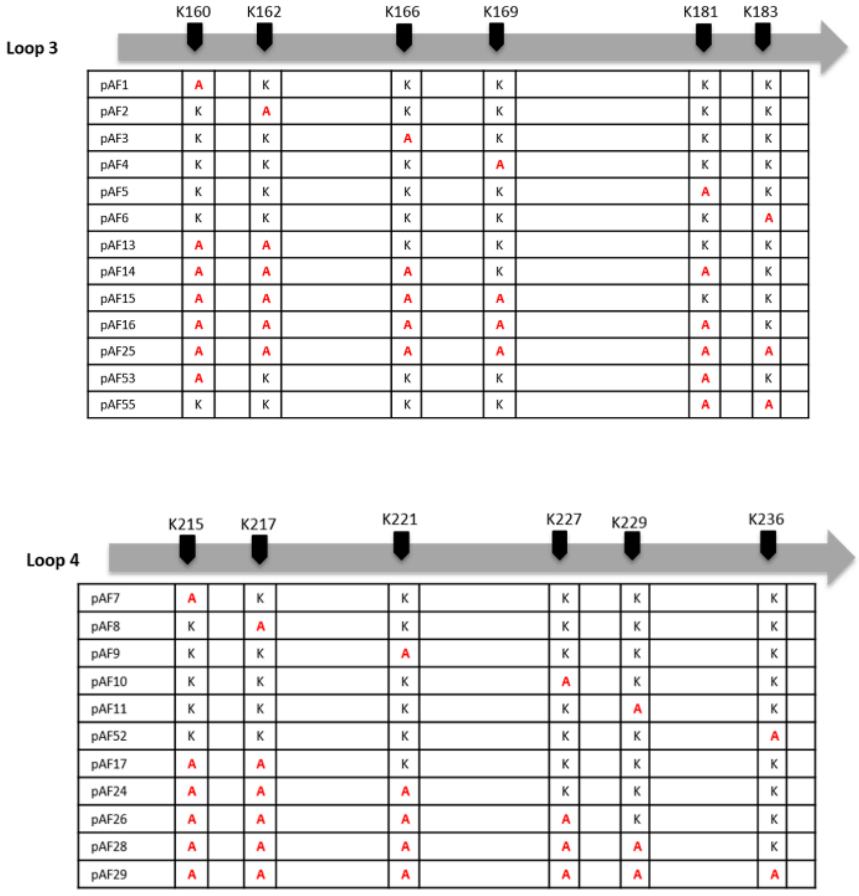

Figure 2.3. Schematic representation of K-A substitutions in *R. conorii* Adr1 loops 3 and 4. The position of each substituted lysine in loops 3 and 4 and the corresponding name of the plasmid is depicted above and verification of expression of Adr1 is verified using anti-Adr1. Data are representative of at least 3 replicates.

We initially verified expression of each construct by transforming a serum-sensitive strain of *E. coli* BL21 (DE3) with the indicated plasmid and inducing protein expression as described above. Western immunoblotting with anti-Adr1 antibodies confirmed the expression of each construct (Figure 2.4 and 2.5). Constructs pAF15, pAF16, pAF25, pAF28 and pAF29 did not express under any condition tested and were not further utilized. Because our data demonstrated that Adr1-Vn interactions are electrostatic in nature and that the Vn-interacting domains of Adr1 (loops 3 and 4) contain a high concentration of positively charged amino acids, we hypothesized that removal of one or more of the positive charge would decrease the ability of Adr1-expressing *E. coli* to survive when exposed to serum and to interact with Vn. To initially determine serum survival, each Adr1 mutant was expressed in *E. coli* and incubated in 10% NHS in PBS and PBS alone as a control. The colony forming units (CFU) of bacteria exposed to NHS were quantified in comparison to the bacteria exposed to PBS and bacterial survival was expressed as the percent bacteria remaining after exposure to serum. As shown in Figure 2.6A and D, Adr1 mutants containing a single lysine to alanine substitution in any position within each loop remained resistant to serum killing. In contrast, when the first two or more positive charges to uncharged substitutions were made, the ability of *E. coli* expressing Adr1 mutant proteins to resist serum killing was significantly decreased (Figure 2.6B, 2.6C, 2.6E and 2.6F). These results suggest that the first two lysines in either loops 3 or 4 are critical to mediate resistance to serum mediated killing, but do not exclude the possibility that the net charge in either Adr1 loop contributes to the observed phenotype. We, therefore, designed experiments to distinguish between these two possibilities. Using the Adr1-loop 3 construct as a model, we created double lysine mutants at lysine positions 1 and 5 or at positions 5 and 6 (Figure 2.3), expressed these mutant Adr1 proteins in *E. coli,* and tested for their ability to survive in normal human serum. As shown in Figure 2.7

E. coli expressing the indicated lysine to alanine substitutions in Adr1 were able to survive when exposed to human serum. Taken together, these data demonstrate that the first two lysine residues loop 3 are critical to mediate resistance to complement mediated killing and suggests that these residues may play important roles in the interaction with vitronectin.

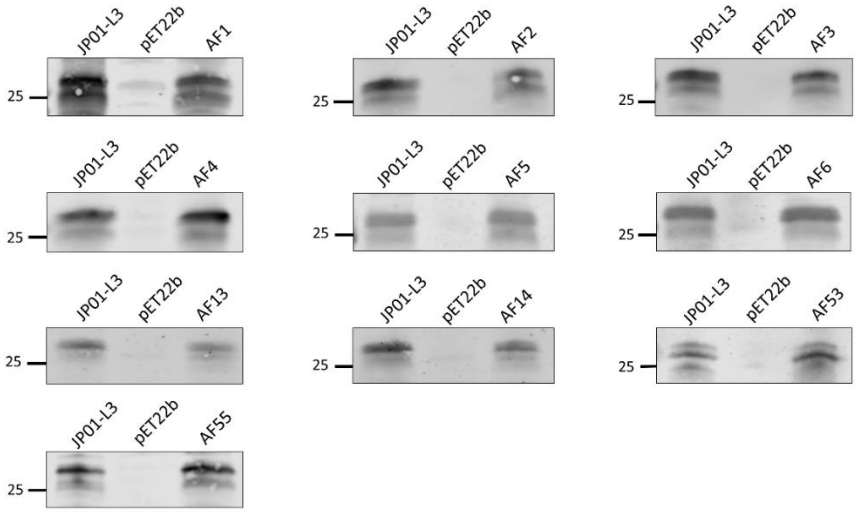

Figure 2.4. Expression of Adr1 loop 3 mutants in *E. coli* BL21(DE3). Western immunoblot analysis of whole cell lysates for each mutant constructed within loop 3 using anti-Adr1.

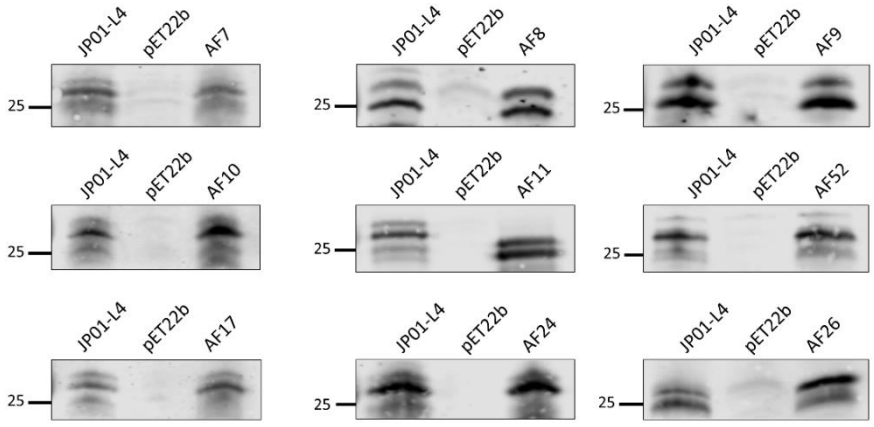

Figure 2.5. Expression of Adr1 loop 4 mutants in *E. coli* BL21(DE3). Western immunoblot analysis of whole cell lysates for each mutant constructed within loop 4 using anti-Adr1.

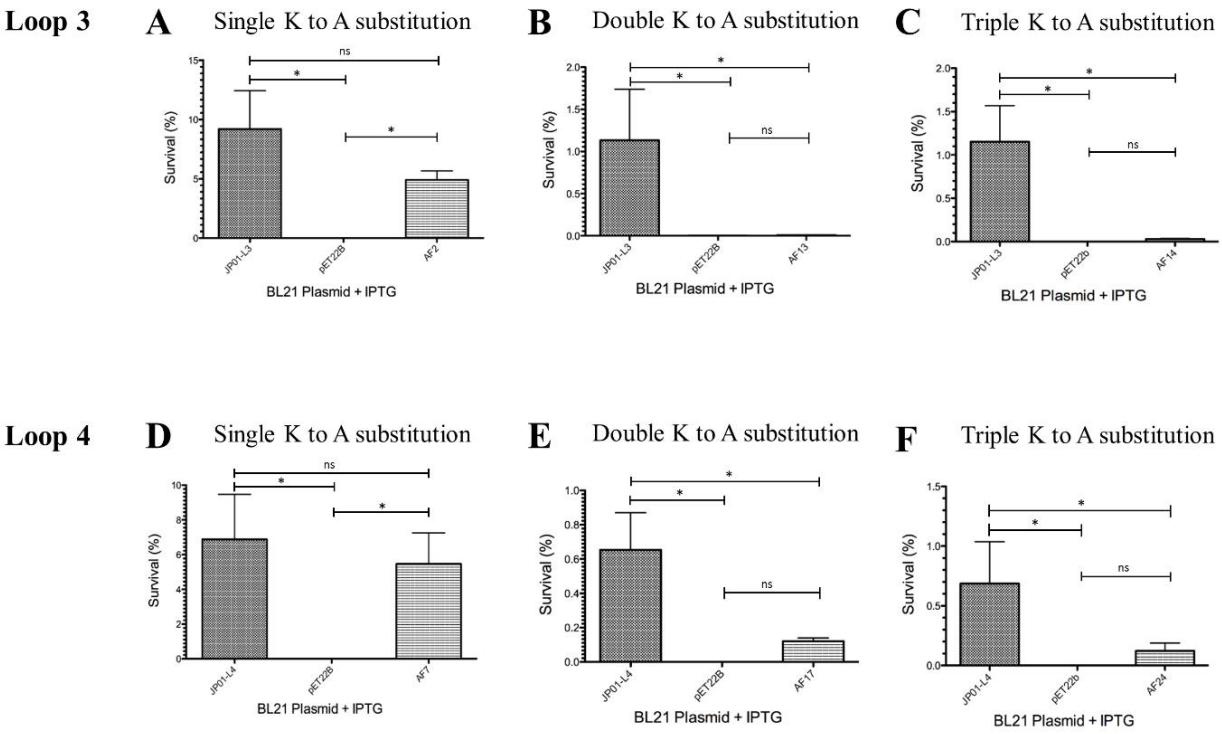

Figure 2.6. Analysis of loop 3 and 4 mutant Adr1 serum resistance when expressed on the surface of *E. coli* and exposed to NHS. A & D. A single lysine to alanine substitution (AF2 and AF7). B & E. A double lysine to alanine substitution at positions 1 and 2 (AF13 and AF17). C & F. A triple amino acid substitution at positions 1, 2 and 3 (AF14 and AF24). Data are representative of at least 3 replicates. A one-way ANOVA with a Newman-Keuls post hoc test was performed. * represent a $p \leq 0.05$ and is considered significant. ns represents no significance.

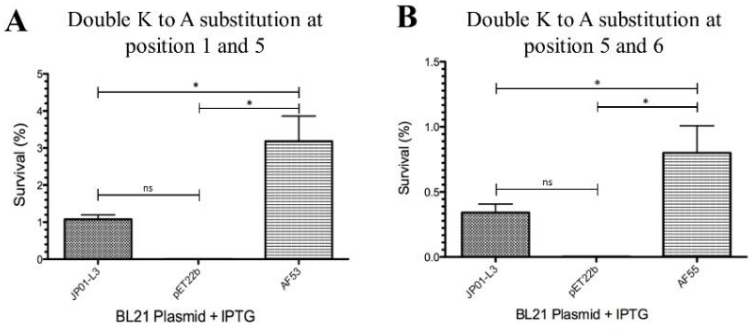

Figure 2.7. Analysis of double mutants of loop 3 Adr1 serum resistance when expressed on the surface of *E. coli* and exposed to NHS. A. A double lysine to alanine substitution at positions 1 and 5 (AF53). B. A double lysine to alanine substitution at positions 5 and 6 (AF55). Data are representative of at least 3 replicates. A one-way ANOVA with a Newman-Keuls post hoc test was performed. * represent a $p \leq 0.05$ and is considered significant. ns represents no significance.

2.3.4 Binding of multimeric Vitronectin is correlated with survival of Adr1-expressing bacteria

We next sought to verify that the serum-resistant phenotypes are correlated with the ability of Adr1 to bind vitronectin. We hypothesized that *E. coli* expressing Adr1 mutants that survived serum killing were sufficient to bind vitronectin, while *E. coli* expressing Adr1 mutants that did not survive in serum were either unable to bind or are greatly impaired in their ability to bind vitronectin. To test this hypothesis, we transformed *E. coli* BL21(DE3) with the constructs for serum resistant Adr1 loop 3 proteins harboring a single amino acid substitution construct, representing serum resistant phenotypes (pAF1 and pAF2), the double amino acid constructs at positions 1 and 5 (pAF53) and at position 5 and 6 (pAF55). Plasmids encoding for serum-sensitive phenotypes were also transformed and include double substitution at positions 1 and 2 construct (pAF13) and triple substitution at positions 1, 2, and 3 construct (pAF14), representing the serum sensitive phenotype. Adr1 loop 4 mutant proteins containing a single amino acid substitution (pAF8, serum resistant) and the double and triple mutants (pAF17 and pAF26, serum-sensitive) were also expressed in *E. coli*. We initially verified the expression of Adr1 mutants at the outer membrane of *E. coli* when expressed under these conditions (Figure 2.8). These bacteria were incubated with NHS and analyzed for their abilities to interact with human Vn via a Western immunoblot analysis. Surprisingly, *E. coli* expressing either serum-resistant or serum-sensitive Adr1 mutant constructs were able to bind Vn in serum (Figure 2.9A and 2.9B).

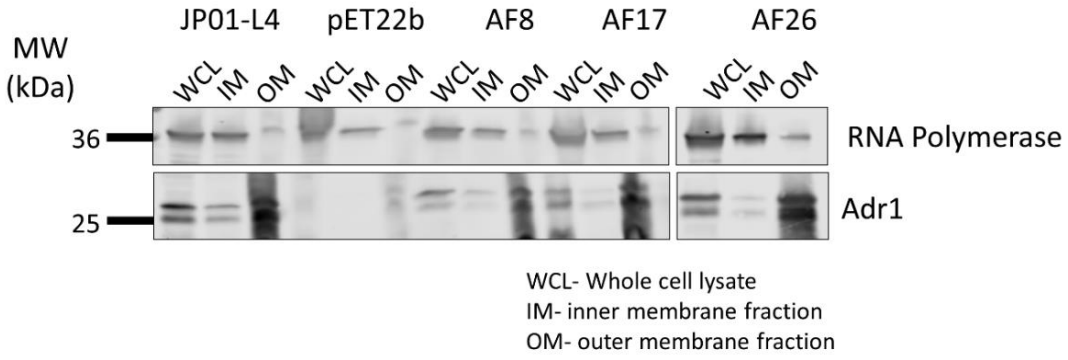

Figure 2.8. Confirmation of expression of mutant Adr1 proteins at the outer-membrane of *E. coli*. Western immune blot analysis of whole cell lysates (WCL), sarkosyl soluble/inner membrane proteins (IM) and outer membrane fractions (OM). An Adr1 derivative containing only loop 4 (JP01-L4) was used as a positive control and the empty vector (pET22b) was used as the negative control. Representative constructs of serum resistant (AF8) and serum-sensitive (AF17) and phenotypes are presented.

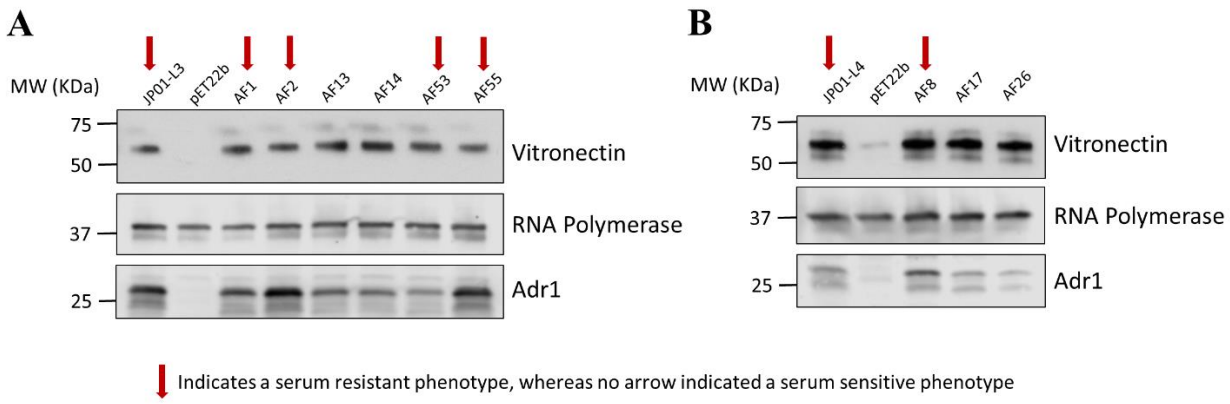

Figure 2.9. Serum resistance or sensitivity does not correlate with the ability to bind vitronectin in serum. A & B. Western immunoblot analysis of vitronectin in serum binding to *E. coli* expressing loop 3 or loop 4 Adr1 mutants with both serum resistant and serum-sensitive phenotypes. Equal loading was verified using anti-*E. coli* RNA polymerase and Adr1 expression was verified using anti-Adr1. Data are representative of at least 3 replicates. Arrows depict Adr1 constructs that confer resistance to serum killing.

Vitronectin within the blood exists in both a monomeric and multimeric form. Consequently, Vn interactions that are responsible for Adr1-mediated serum resistance may in part be dependent on the form with which Adr1 interacts. Because the vitronectin multimer is the functionally active form we hypothesized that serum resistant mutants were able to bind to the multimeric form of Vn while serum-sensitive mutants were unable or significantly reduced in the ability to bind to the multimer. We also hypothesized that all mutants representing both serum-resistant and sensitive phenotypes would bind to the vitronectin monomer. To test our hypotheses, the aforementioned constructs were expressed at the outer membrane of *E. coli*. The bacteria were then incubated with monomeric and multimeric vitronectin and analyzed for the ability to bind to each form. The results of our experiment demonstrated that serum-resistant mutants bound to multimeric vitronectin (Figure 2.10A and 2.10C) whereas serum-sensitive mutants had a decreased ability to bind to the multimer. Conversely, all mutant regardless of phenotype were able to sufficiently bind to monomeric vitronectin (Figure 2.10B and 2.10D). These data indicate that electrostatic Adr1-Vn interactions are important for serum resistance and specifically serum resistance can be correlated with the ability of the bacteria to bind to the multimeric form of human vitronectin.

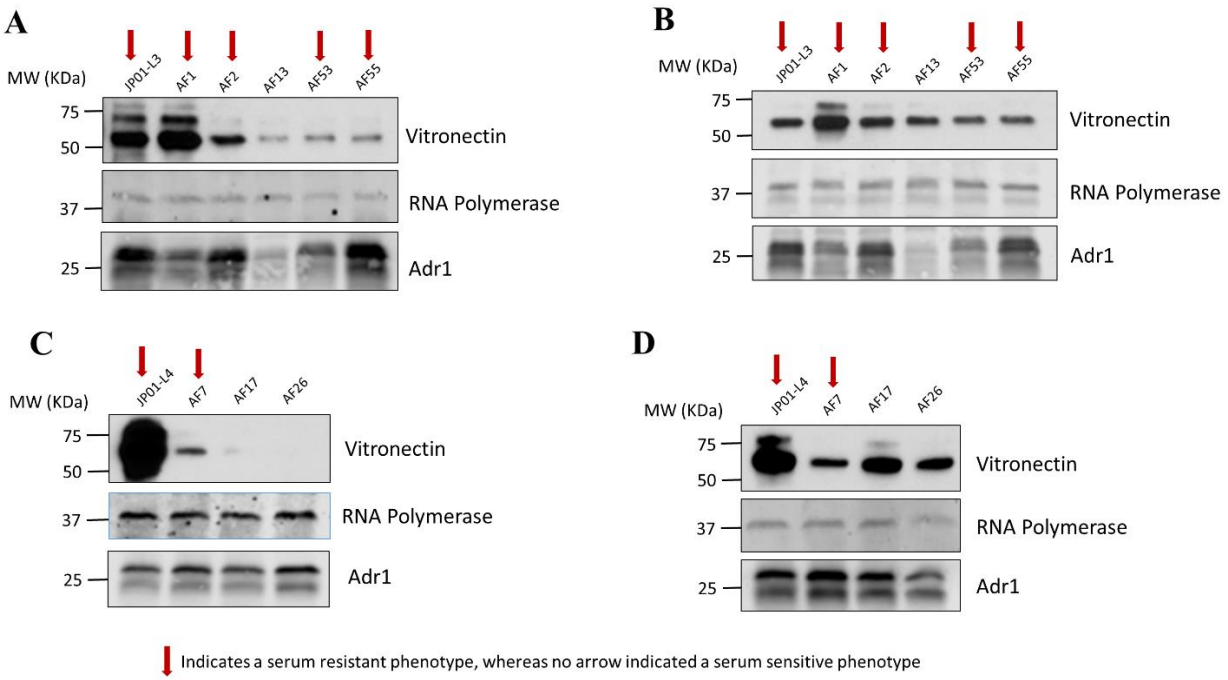

Figure 2.10. Analysis of monomeric and multimeric vitronectin binding to loop 3 and 4 mutants. A & C. Western immunoblot analysis of multimeric vitronectin binding to *E. coli* expressing loop 3 or loop 4 Adr1 mutants with both serum resistant and serum-sensitive phenotypes. B & D. Western immunoblot analysis of monomeric vitronectin binding to *E. coli* expressing loop 3 or loop 4 Adr1 mutants with both serum resistant and serum-sensitive phenotypes. Equal loading was verified using anti-*E. coli* RNA polymerase and Adr1 expression was verified using anti-Adr1. Data are representative of at least 3 replicates. Arrows depict Adr1 constructs that confer resistance to serum killing

2.4 Discussion

A previous report from this laboratory demonstrated that *R. conorii* binds vitronectin (Vn) to facilitate evasion of complement-mediated killing (Riley et al., 2014). Additionally, Adr1 loops 3 and 4 were sufficient to mediate the interaction with Vn. In the present study, we focused on elucidating the molecular determinants of the Adr1/vitronectin protein/protein interaction. Human vitronectin is a multifunctional human glycoprotein that is part of both the extracellular matrix and found in plasma. This protein plays a role in many biological functions including cell

migration, tissue repair, and regulation of membrane attack complex formation by binding C5b-C7 complex and inhibition of C9 deposition on the surface of a cell or microbe (Singh et al., 2010b; da Silva et al., 2015). Vitronectin has a multi-domain structural arrangement that consists of an N-terminal somatomedian B domain which binds plasminogen activator inhibitor-1 and an RGD (arginine, glycine and aspartic acid) domain that interacts with several integrins thereby aiding in attachment (Blom et al., 2009). Vitronectin also contains three heparin-binding domains and a C-terminal region with an unknown function, both of which have been shown to bind to surface proteins of bacterial pathogens (Blom et al., 2009; Singh et al., 2010a; Singh et al., 2011). Homologs to human vitronectin are found among many mammalian species including rabbits, mice, and cows. Interestingly, when these mammalian vitronectin proteins are compared there are regions of variability in both the N- and C-termini. Human vitronectin contains several unique residues within the C-terminal region, which may play an important role in protein-protein interactions (Leduc et al., 2009; da Silva et al., 2015). Because vitronectin is one of the major regulators of membrane attack complex formation, it plays a critical role in deposition of the MAC on bacterial pathogens. Gram-negative pathogens including *R. conorii*, *Legionella pneumophila*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *M. caterrhalis*, and *H. influenzae* utilize vitronectin to protect against MAC killing (Hallstrom et al., 2006; Hallstrom et al., 2009; Singh et al., 2010a; Riley et al., 2014; Hallstrom et al., 2016). On the other hand, Gram-positive bacteria such as *S. pneumoniae* and *S. pyogenes* utilize vitronectin as a bridge to bind to and invade host cells (Leroy-Dudal et al., 2004; Singh et al., 2010b).

Our results indicate that the Adr1/Vn interaction is a heparin-independent, electrostatic interaction based on the ability of increasing concentrations of NaCl, but not heparin to inhibit the interaction between the two proteins. The ability of the recombinant peptide that contains a

deletion within the C-terminal heparin binding domain (352-362) to bind to Adr1 when expression on the surface of *E. coli* further supports our conclusion that the Adr1/Vn interaction is heparin-independent. To our knowledge, this is the first demonstration of an outer-membrane protein in a Gram-negative bacterial pathogen that interacts with vitronectin in a heparin-independent, salt-sensitive manner. Heparin-dependent interactions, on the other hand, are well documented in Gram-negative pathogens (Hallstrom et al., 2016) and include proteins such as Ubiquitous surface protein A2 (UspA2) *of M. catarrhalis, Haemophilus* surface fibrils (Hsf) of *H. influenzae* type B, and Protein E (PE) and Protein F (PF) from non-typable *H. influenzae* (NTHi) (Hallstrom et al., 2006; Singh et al., 2010a; Singh et al., 2011; Su et al., 2013). Interestingly, a Gram-negative pathogen, *N. meningitidis*, interacts with vitronectin via Meningococcal surface fibrils (Msf) in a heparin-independent manner; however, salt dependence has yet to be determined (Griffiths et al., 2011). Similarly, to the Adr1/vitronectin interaction in *R. conorii*, the Gram-positive pathogen, *S. pneumoniae* interacts with vitronectin via Pneumococcal surface protein C (PspC) in a salt sensitive manner, signifying an electrostatic interaction. Increasing concentrations of salt competitively inhibited the ability of vitronectin to bind to PspC suggesting that the negatively charged amino acids in PspC mediate this interaction; however, individual amino acids responsible for this interaction have yet to be identified (Voss et al., 2013). The Adr1/Vn interaction is different from other bacterial/Vn interactions and is likely unique to the Rickettsiales.

We utilized recombinant vitronectin peptides to elucidate the Vn region(s) required for association with Adr1. This region (amino acids 312-396) plays an important role in binding surface exposed proteins of both Gram-negative and Gram-positive bacterial pathogens (Singh et al., 2010a; Singh et al., 2011; Su et al., 2013; Voss et al., 2013; Hallstrom et al., 2016). Like many other bacterial pathogens, Adr1 from *R. conorii* was demonstrated to bind within the C-terminal

region of vitronectin (amino acids 363-373), adjacent to the third heparin-binding domain. In contrast, many gram-negative and gram-positive bacteria including *S. pneumoniae, H. influenzae P. aeruginosa, Staphylococcus aureus, S. pyogenes* all of which have heparin-dependent interactions with vitronectin, bind within a domain (amino acids 352 and 374) containing a portion of the third heparin-binding domain (Hallstrom et al., 2006; Singh et al., 2011; Voss et al., 2013; Hallstrom et al., 2016). *H. influenzae* also utilizes a heparin-mediated interaction with vitronectin, but the region required for binding to PE encompasses a longer sequence (amino acids 353-396) (Hallstrom et al., 2009; Hallstrom et al., 2016).

A few other electrostatic protein-vitronectin interactions have been described. For example, the Gram-positive pathogen, *S. pneumoniae* utilizes PspC to bind vitronectin in a salt-sensitive manner. Although individual amino acids responsible were not identified, researchers suspect the negatively charged amino acids of the R domain in PspC mediate this interaction (Voss et al., 2013). In addition, the Gram-negative pathogen, *H. influenzae* binds vitronectin via PE in a heparin-dependent manner; however, two amino acids, leucine 85 and arginine 86 have been identified as critical for this interaction (Singh et al., 2011). Although leucine is an uncharged non-polar residue, arginine is positively charged and this charge could possibly contribute to the interaction. We determined that the Adr1/vitronectin interaction was an electrostatic interaction that was not mediated by a single charged residue. Instead, we observed that a substitution in the first two lysine residues in loop 3 and 4 caused a significant decrease the inability of *E. coli* expressing these proteins to evade the bactericidal effects of normal human serum. It is possible that these 2 amino acids in each loop independently create a critical initial interaction domain that is necessary for stable Adr1-vitronectin interactions and that without these two residues, Adr1 is not able to effectively mediate this protein-protein interaction. This hypothesis is further supported

by the observation that perturbing the overall charge in Adr1 loop 3 had no deleterious effect on the ability of *E. coli* expressing this protein to survive serum-mediate killing.

Surprisingly, our results did not demonstrate a direct correlation between the ability to bind Vn and the ability to mediate survival in serum. Within human serum, vitronectin exists in both a monomeric and a multimeric form. The predominant form in plasma is a monomer and is also referred to as the "native" form (Stockmann et al., 1993). Multimeric vitronectin is found in small quantities, and consists of intra-molecular interactions between monomers (Stockmann et al., 1993). Previous studies examining vitronectin binding to outer-membrane proteins of various other pathogenic bacteria have looked at the native versus active form. The native form of vitronectin is folded, whereas the active form is conformationally altered and has an "open" structure, meaning that certain cryptic epitopes are available that are likely masked in the native form (Sa et al., 2010). Human serum is predicted to contain more than 7% of the activated form of vitronectin (Sa et al., 2010). Interestingly, the outer membrane protein, Opc of *N. meningitidis* exhibits preferential binding to the active form of vitronectin to mediate adhesion and invasion of brain cells (Sa et al., 2010). Conversely, *Yersinia pestis* utilizes two outer membrane proteins, Ail and Pla, to bind and proteolytically process the native form of vitronectin to facilitate resistance to complement mediated killing (Bartra et al., 2015). Upon further testing, we were demonstrated that serum-resistant Adr1 mutants bound both multimeric and monomeric Vn whereas serum-sensitive mutants bound to monomeric Vn, but were substantially impaired in their ability to bind to multimeric Vn. The data suggests that multimeric vitronectin mediates protection from complement-mediated killing. Therefore, vitronectin interactions that are responsible for Adr1-mediated serum resistance are dependent on the form with which Adr1 interacts. It is apparent

that the critical Adr1-vitronectin interaction that contribute to *R. conorii* survival in serum is much more complex than originally thought.

Recent analysis of patients with confirmed cases of *R. conorii* infection demonstrated increased serum concentration of complement activation markers (Otterdal et al., 2016). The logical conclusion from those studies is that *R. conori* infection activates complement, potentially leading to release of inflammatory cytokines and chemokines, and increases monocyte activation (Otterdal et al., 2016). While this data may appear to be contrary to our findings, a cursory glance at the complement cascade demonstrates that this is not the case. Herein and in previous publications, we describe rickettsial mechanisms for avoiding killing in serum. The Adr1/Vn interaction simply implies that, regardless of the state of complement activation, *R. conorii* is able to prevent activation and deposition of the antibacterial terminal complement complex. As such, the findings of Otterdal, et.al. dovetail with our results, because *in vivo* complement activation indicates that *R. conorii* must avoid complement-mediated killing in order to establish (Riley et al., 2012; Riley et al., 2014).

Neisseria meningitidis is a human pathogen that causes increased morbidity and mortality worldwide as a result of sepsis and meningitis. Like *R. conorii*, *N. meningitidis* has the ability to bind to complement regulatory proteins in order to avoid complement mediated killing. Specifically, this bacteria binds Factor H via factor H-binding protein to avoid deposition of C3b and activation of the alternative arm of the complement system (Madico et al., 2006). When antibodies were created against this protein, the ability of the bacteria to bind Factor H was significantly decreased and as a result, the bacteria were not able to survive in serum (Madico et al., 2006). This discovery lead to the development of a preventative vaccine against multiple strains of *N. meningitidis* directed against factor H-binding protein (Seib et al., 2015; Gandhi et

al., 2016). Herein, we demonstrate the molecular determinants of the interaction between *R. conorii* Adr1 and human Vn. The results gathered from this work further contribute to our understanding of the molecular mechanisms by which pathogenic rickettsial species establish successful infections in mammalian hosts. Elucidation of the interacting interface(s) in Adr1 and vitronectin will hopefully better guide the development of novel and efficacious anti-rickettsial therapies that specifically target this important protein-protein interaction.

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CHAPTER 3
RICKETTSIA CONORII EXPLOITS VITRONECTIN FOR INVASION OF UNDIFFERNTIATED THP1 CELLS

3.1 Introduction

Spotted Fever Group (SFG) *Rickettsia* are Gram-negative obligate intracellular bacteria that are responsible for a number of emerging infectious diseases. Members of this group include *R. rickettsii* and *R. conorii*, the etiologic agents of Rocky Mountain Spotted Fever (RMSF) and Mediterranean Spotted Fever (MSF), respectively (Parola et al., 2005). These pathogens are transmitted to a mammalian host through tick salivary contents when an arthropod vector takes a blood meal (Hackstadt, 1996). Upon inoculation into the vertebrate host, the bacteria spread throughout the body via the bloodstream and parasitize cells of many origins including monocytes, macrophages, endothelial cells and hepatocytes (Walker et al., 1994; Walker, 1997; Walker et al., 1999; Curto et al., 2016). Infection of endothelial cells by SFG *Rickettsia* can lead to disruption of the endothelial lining resulting in fluid leakage into the interstitial space causing the characteristic dermal rash (Walker and Ismail, 2008). If left untreated, infection can result in renal failure, non-cardiogenic pulmonary edema, interstitial pneumonia and other multi-organ manifestations (Raoult and Roux, 1997). When the infection is properly identified and treated promptly, prognosis is good; however, misdiagnosis is common due to initial non-descript flu-like symptoms resulting in increased morbidity and mortality (Chan et al., 2009; Riley et al., 2014).

Interaction of the bacterium with host cells is a critical step in the establishment of infection as *rickettsia* have evolved to be completely dependent on the nutrient rich cytoplasm of the host cell and are not thought to replicate extracellularly (Chan et al., 2010). The process begins with recognition and adherence of the bacteria with a targeted host cell through an array of bacterial surface proteins and specific eukaryotic cell surface receptors. Binding of the bacteria to a host

cell ligand triggers a signaling cascade within the cell which ultimately leads to bacterial internalization (Martinez and Cossart, 2004). Morphologically and mechanistically this process resembles a zipper-like invasion strategy whereby the receptor/ligand interaction induces actin cytoskeleton rearrangement at the site of interaction causing "zippering" of the membrane around the bacterium and ultimately engulfment (Gouin et al., 1999).

To date, a small number of rickettsial proteins have been identified that contribute to adherence and invasion of mammalian cells including outer membrane protein A (OmpA), outer membrane protein B (OmpB), surface cell antigen 1 (Sca1) and surface cell antigen 2 (Sca2) (Li and Walker, 1998; Martinez and Cossart, 2004; Cardwell and Martinez, 2009; Chan et al., 2009; Riley et al., 2010; Hillman et al., 2013). These proteins are all members of a highly conserved family of genes within SFG *Rickettsia* termed surface cell antigens (*sca*) and encode for outer membrane proteins (Blanc et al., 2005). Importantly, the predicted structures of the aforementioned proteins share homology with a family of proteins present in Gram-negative bacteria termed autotransporters, many of which are known virulence factors (Henderson and Nataro, 2001). In *R. conorii*, Sca1, Sca2, OmpA and OmpB have all been demonstrated to facilitate adherence to non-phagocytic mammalian cells *in vitro*; however, only Sca2, OmpA and OmpB are sufficient to mediate internalization (Cardwell and Martinez, 2009; Chan et al., 2009; Riley et al., 2010; Hillman et al., 2013). Previous studies have also identified the mammalian ligands of OmpA and OmpB as the $\alpha_2\beta_1$ integrin, fibroblast growth factor receptor-1 (FGFR1) and Ku70 (Martinez et al., 2005; Hillman et al., 2013; Sahni et al., 2017). Aside from these studies, very little is known about the identity of other mammalian proteins that can serve as receptor for this class of obligate intracellular bacteria.

Another protein expressed at the surface of *R. conorii*, termed Adr1, is also suggested to be a putative adhesion because it was demonstrated to associate with an unknown mammalian protein *in vitro* and because antibodies directed against this protein inhibited interactions with mammalian cells (Renesto et al., 2006; Balraj et al., 2009). The rickettsial Adr1 proteins from various species of *rickettsia* differ in amino acid sequences but are all predicted to contain eight outer-membrane spanning β strands and four extracellular domains termed "loops." Several proteins that serve as adhesins in other Gram-negative bacteria are also predicted to adopt this structure. A recent study on *R. conorii* Adr1 demonstrated that the protein interacts with the multifunctional human glycoprotein vitronectin (Riley et al., 2014). Vitronectin is an extracellular matrix protein and is important for the attachment of cells to the surrounding matrix, regulation of cell differentiation, proliferation and morphogenesis, and exerts regulatory function on blood coagulation and the complement cascade (Preissner and Seiffert, 1998). It exists as both an internally folded and stabilized monomer and a partially unfolded multimer (Schvartz et al., 1999). Generally, monomeric vitronectin is found in high concentrations in circulating plasma while multimeric vitronectin is located in the extracellular matrix (Preissner and Seiffert, 1998). Interaction of vitronectin with different chaotropic agents and macromolecules has been described to induce a conformational transition from monomeric to the reactive multimeric complex (Zhuang et al., 1996). Interestingly, many pathogenic bacteria, including pathogenic rickettsial species, have evolved mechanisms to utilize vitronectin to evade complement-mediated killing (Riley et al., 2014). Further characterization of *R. conorii* Adr1-vitronectin interactions revealed that both the monomeric and multimeric form of vitronectin bound to Adr1. This study also revealed that *R. conorii* interactions with the multimeric form and not the monomeric form of vitronectin correlated with protection against serum killing (Fish et al., 2017). These findings led to further

investigations regarding the significance of *R. conorii*-monomeric vitronectin interactions in the infection process. Interestingly, vitronectin can often serve as a "bridge" between pathogenic bacteria and target host cells in mediating adherence and invasion of target host cells (Duensing and van Putten, 1997; Gomez-Duarte et al., 1997; Dehio et al., 1998; Leroy-Dudal et al., 2004; Bergmann et al., 2009; Sa et al., 2010; Al-Jubair et al., 2015). Therefore, it is hypothesized that similar to other pathogens, acquisition of monomeric vitronectin may facilitate rickettsial adherence and invasion into a variety of target mammalian cells. It has previously been demonstrated *in vivo* and *in vitro* that pathogenic rickettsial species interact with several cell types including endothelial cells, circulating monocytes, neutrophils, lymphocytes and resident macrophages (Walker and Gear, 1985; Walker et al., 1994; Walker, 1997; Walker et al., 1999; Banajee et al., 2015; Curto et al., 2016; Riley et al., 2016). Whereas several studies have highlighted the molecular events that govern interactions with endothelial cells, nothing is known about the mechanisms by which rickettsial species interact with other non-endothelial cell types. To model these interactions *in vitro*, undifferentiated THP-1, differentiated THP-1 and EA.hy cells representing human monocytes, macrophages and endothelial cells, respectively, were utilized. It was hypothesized that initial exposure of *R. conorii* to vitronectin in the hematogenous circulation would not only be important for resistance to serum-mediating killing, but also may play a role in facilitating interactions with endothelial cells and circulating monocytes. Herein, the role of monomeric and multimeric vitronectin binding by *R. conorii* in adherence and invasion of specific cells in *in vitro* models of infection was analyzed.

3.2 Materials and Methods

3.2.1 Cell Lines, *Rickettsia* Growth and Purification

EA.hy926 (ATCC® CRL-2922™) cells (somatic cell hybrid endothelium) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals), 1x non-essential amino acids (Corning) and 0.5 mM sodium pyruvate (Corning). THP-1 (ATCC® TIB-202™) cells (human peripheral blood monocytes) were grown in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum. Differentiation of THP-1 cells into macrophage-like cells was carried out by the addition of 100 nM of phorbol 12-myristate 13-acetate (PMA; Fisher) to $2x10^5$ cells/mL. Cells were allowed to differentiate and adhere for 3 days prior to experimentation. All cells were maintained in a humidified incubator at 37°C in 5% CO_2. *R. conorii* Malish7 was propagated in Vero cells and purified as previously described (Ammerman et al., 2008; Chan et al., 2009; Chan et al., 2011).

3.2.2 Antibodies and Reagents

Anti-Rc_{PFA} rabbit polyclonal antibody that recognizes *R. conorii*, was generated as previously described (Chan et al., 2011). Mouse anti-bovine vitronectin was purchased from LifeSpan Bioscience, Inc and sheep anti-human vitronectin was obtained from AbD Serotec. Secondary antibodies, Alexa-Fluor 488- and 546-conjugated goat anti-rabbit IgG, DAPI, and Far Red-X-phalloidin were purchased from Thermo Scientific. IRDye® 680 Donkey anti-mouse IgG (H+L) was acquired from LI-COR Biosciences and IRDye® 800CW Donkey anti-Sheep IgG (H+L) was purchased from Rockland Antibodies and Assays. Multimeric and Monomeric human vitronectin were purchased from Innovative Research.

3.2.3 Vitronectin Binding Assay

To evaluate vitronectin adherence to the surface of each cell line: EA.hy926 cells were seeded into six wells of a 24-well plate at $1x10^5$ cells/well 24 hours prior to experimentation; differentiated THP-1s were seeded at $2x10^5$ cells/well 3 days prior to experimentation; and undifferentiated THP-1 cells were suspended in 1 mL of serum free media at a density of $2x10^5$ cells/mL immediately prior to experimentation. On the day of the experiment, cells were washed five times with PBS to remove any associated proteins from the growth media with the surface of the cells. One well of each plate containing EA.hy926 or differentiated THP-1 cells were then exposed to either serum free media, complete media, or serum free media containing 2 or 4 μg/mL multimeric or monomeric vitronectin. Undifferentiated THP-1 cells were centrifuged at 500 x g for 5 minutes and resuspended in each of the aforementioned treatments. All cells were incubated at 34ºC with 5% CO_2 for 30 minutes and then washed three times with PBS. Adherent cells were lifted from the tissue culture flask by scraping in 1 mL PBS and the cells were then transferred to 1.5 mL microcentrifuge tubes. All cells were pelleted and total cellular lysates were prepared (Martinez et al., 2005). Binding of bovine and human vitronectin was determined by SDS-PAGE and Western immunoblotting using mouse anti-bovine vitronectin and sheep anti-human vitronectin primary antibodies and donkey anti-mouse IgG 680 donkey and anti-sheep IgG 800 secondary antibodies followed by densitometry analysis on a LI-COR Odyssey CLx system.

3.2.4 Bacterial Association Assay

To assess if vitronectin binding to the cellular surface increases the ability of *R. conorii* to adhere to specific cell types, the following experimental scheme was utilized: EA.hy926 cells were seeded onto sterile glass coverslips (Fisher) in 24-well plates at $1x10^5$ cells/well 24 hours prior to experimentation; differentiated THP-1 cells were seeded onto glass coverslips in a 24-well plate

at a density of $2x10^5$ cells/well 3 days prior to experimentation; and undifferentiated THP-1s were suspended in 1 mL of serum free media at a density of $2x10^5$ cells/mL prior to experimentation. In order to determine if cell bound vitronectin increased adherence of *R. conorii* to the cellular membrane, cells were washed five times with PBS to remove any proteins associated with the outer-membrane from the growth media. Cells were then exposed to 1mL serum free media or serum free media contain 2 or 4 μg/mL multimeric or monomeric vitronectin and incubated for 30 minutes at 34ºC with 5% CO_2. After 30 minutes, the cells were then washed 3 times with PBS and cells were infected with *R. conorii* at a multiplicity of infection (MOI) of 10. The plates were centrifuged at 300 x g for 5 minutes at room temperature to induce bacterial contact with the cells and bacteria were allowed to bind for 30 minutes in a humidified incubator at 34ºC. Undifferentiated THP-1 cells were pelleted post vitronectin treatment and resuspended in 1 mL serum-free media containing *R. conorii* at an MOI of 10. Cells were then incubated at 34ºC with agitation for 30 minutes. All cells were washed 2 times with PBS and adherent cells were fixed for 20 minutes with 4% paraformaldehyde. Undifferentiated THP-1s were mounted on slides using a Cytospin system and subsequently fixed. To determine if vitronectin bound to *R. conorii* increased adherence to the cell surface, *R. conorii* was suspended in serum free media or serum free media containing 2 or 4 μg/mL of multimeric or monomeric vitronectin. The rickettsiae were then incubated at 34ºC with agitation for 30 minutes. After 30 minutes, the bacteria were washed 3 times with PBS and resuspended in 3 mL of serum-free media. Cells were washed and 1 mL of each vitronectin treated *R. conorii* was added to each well. The plates were centrifuged at 300 x G for 5 minutes and were incubated at 34ºC with 5% CO_2 for 30 minutes. Undifferentiated THP-1 cells were resuspended in 1 mL vitronectin treated *R. conorii* and incubated with agitation for 30 minutes. Cells were then washed, fixed and mounted as described above. Thereafter, infected

cell monolayers were processed for immunofluorescence microscopy. Initially, cells were stained with rabbit anti- Rc_{PFA} at 1:1000 in 2% BSA/PBS followed by Alexa Fluor 546-conjugated goat anti rabbit IgG at 1:1000. Then, cells were permeabilized with 0.1% Triton-X 100 in 2% BSA/PBS for 1 hour. After permeabilization, cells were stained with rabbit anti- Rc_{PFA} at 1:1000 in 2% BSA/PBS followed by Alexa Fluor 488-conjugated goat anti rabbit IgG at 1:1000, DAPI at 1:1000 and Texas Red-X-phalloidin at 1:200. Cells were then rinsed with PBS and glass coverslips were mounted in Mowiol semi-permanent mounting medium. Experiments were performed in triplicate and the result of each experiment was expressed as the ratio of *rickettsia* to mammalian cells denoted by nuclei. At least 200 nuclei were counted for each experiment. Preparations were viewed with a Leica DM 4000b compound microscope equipped with an OLYMPUS DP80 camera with final optical zoom of 40x. *Rickettsia* were counted using the cell counter analysis tool from ImageJ provided by the NIH (https://imagej.nih.gov/ij/). Adherence per cell was calculated as the number of *rickettsia* that bound the cellular membrane/number of nuclei. Percent invasion was calculated as extracellular *rickettsia* subtracted from all *rickettsia* to give internal *rickettsia*, which was then divided by total *rickettsia*. Statistical analysis was performed using a one-way Anova with a Dunnet's post-hoc test. Differences were considered significant with a *p*-value less than or equal to 0.05 using Graph-Pad Prism Version 5.0b (GraphPad Software).

3.2.5 Flow Cytometry

Approximately $1x10^6$ undifferentiated THP1, differentiated THP1 and EA.hy926 cells were washed with PBS and fixed with 4% PFA. The expression of $\alpha_V\beta_3$ integrin on the cellular surface was detected using a monoclonal mouse anti- $\alpha_V\beta_3$ integrin (R&D Systems) and secondary Alexa Fluor 488-conjugated goat anti mouse IgG (Thermo Scientific). Cells were analyzed with a FacsCaliber using FITC (488nm excitation 520/30 nm emission) and FloJo software.

3.3 Results

3.3.1 Bovine vitronectin does not adhere to the cell surface, while human vitronectin binds to undifferentiated THP1 and EA.hy926 cells

During routine culture of THP-1 and EA.hy926 cells, the cells are bathed in media containing heat-inactivated fetal bovine serum (FBS). Although the serum components have been denatured and subsequently inactivated, it was prudent to determine if vitronectin from FBS was able to bind to the cellular surface of each cell line and therefore out compete human vitronectin for association with the cell surface. It was hypothesized that bovine vitronectin would not adhere to the surface of the cells because the protein had been denatured while human vitronectin would adhere to undifferentiated THP-1s (monocytes), differentiated THP-1s (macrophage-like), and EA.hy926 (endothelial) cells. To test this hypothesis cells were washed and treated with serum-free media, complete media with FBS or serum-free media containing different concentrations of human monomeric and multimeric vitronectin and probed for both bovine and human vitronectin binding as described above. As depicted in Figure 3.1 A-C, bovine vitronectin did not adhere to the surface of monocytes, macrophages or endothelial cells. Conversely, when human vitronectin binding was accessed, monocytes and endothelial cells were able to bind to both multimeric and monomeric vitronectin, while only minimal amounts of vitronectin adhered to the surface of macrophages (Figure 3.2A-C). Densitometric analysis of vitronectin interacting with host cells demonstrated a significant increase in the ability of monomeric vitronectin to adhere to the surface of monocytes while both multimeric and 4 μg of monomeric vitronectin were able to significantly adhere to endothelial cells (Figure 3.2D-F). Surprising, neither multimeric nor monomeric vitronectin was able to significantly adhere to the surface of macrophages at the indicated concentrations. Taken together, these results demonstrate that vitronectin can associate with the cellular surface of undifferentiated THP-1 cells and EA.hy926 cells, but not differentiated THP1

cells. Therefore, analyses of putative vitronectin-mediated host cell interactions was restricted to EA.hy926 endothelial cells and undifferentiated THP-1 monocytes.

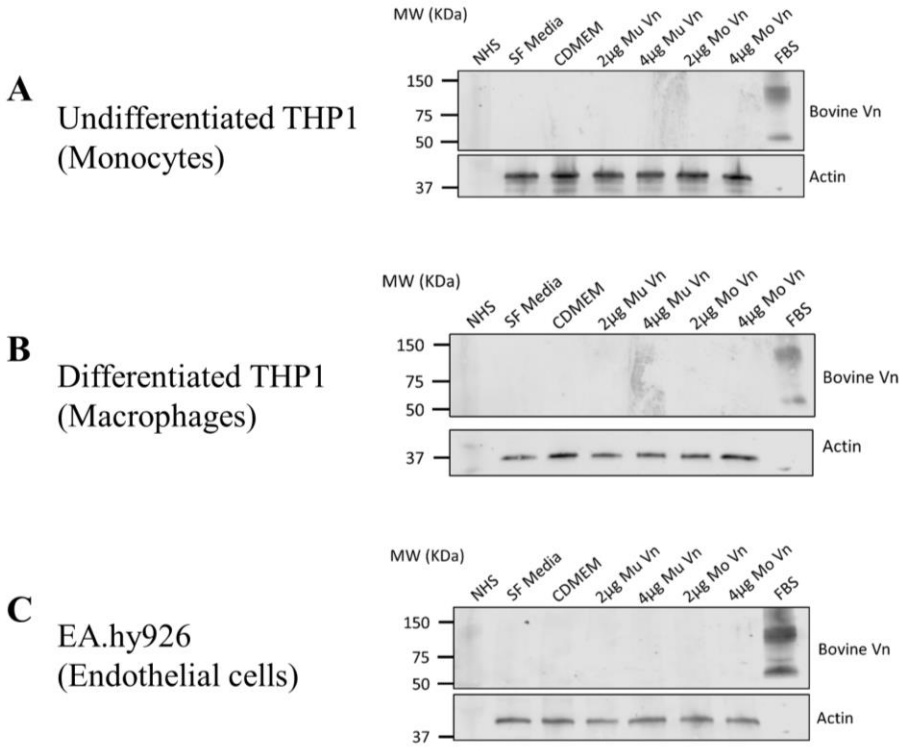

Figure 3.1. Bovine vitronectin binding to monocytes, macrophages and endothelial cells. Western immunoblot analysis of bovine vitronectin binding to monocytes (A), macrophages (B) and endothelial cells (C) when the cells are exposed to FBS, 2 or 4 µg of monomeric (mo) or multimeric (mu) vitronectin (vn). Equal loading in each experiment was verified using an anti-actin antibody. NHS- Normal human serum; CDMEM- complete DMEM; FBS- fetal bovine serum.

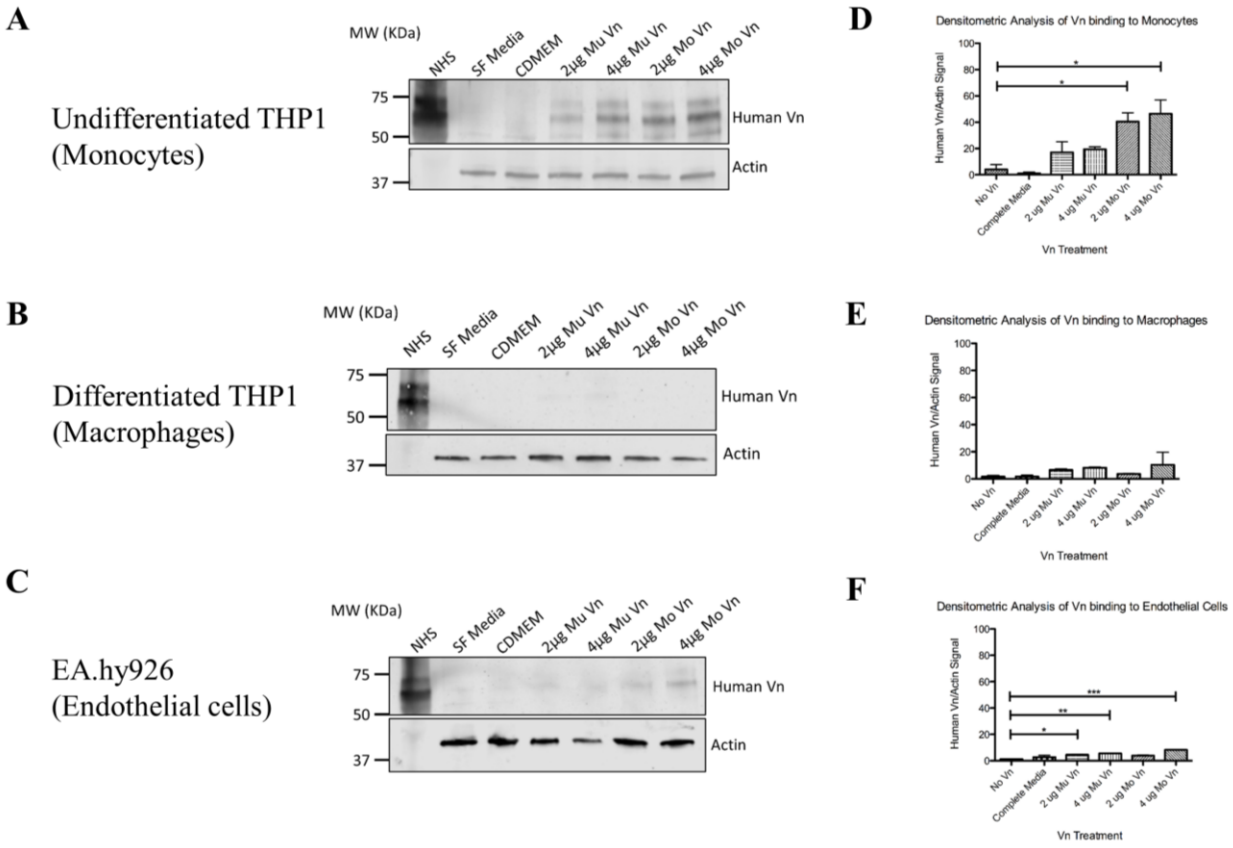

Figure 3.2. Human vitronectin binding to monocytes, macrophages and endothelial cells. Western immunoblot analysis of bovine vitronectin binding to monocytes (A), macrophages (B) and endothelial cells (C) when the cells are exposed to FBS, 2 or 4 µg of monomeric (mo) or multimeric (mu) vitronectin (vn). Equal loading in each experiment was verified using an anti-actin antibody. D, E, & F. Densitometric analysis of vitronectin signal compared to actin control. Data is representative of 2 replicates. A one-way ANOVA with a Dunnet's post hoc test comparing all experimental units to a single control (no vn) was performed. * represents a $p \leq 0.05$ and is considered significant. NHS- Normal human serum; CDMEM- complete DMEM; FBS- fetal bovine serum.

3.3.2 Vitronectin binding by *R. conorii* increases invasion into monocytes

Pathogens such as *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* have been demonstrated to utilize vitronectin as a bridge to increase adherence and invasion of host cells and for evasion of complement mediated killing (Leroy-Dudal et al., 2004; Bergmann et al., 2009; Sa et al., 2010). When rickettsiae are initially inoculated into a human host by an arthropod vector, the bacteria are briefly located extracellularly and likely interact with

endothelial cells of the vasculature and circulating cells such as monocytes. We had previously demonstrated that *R. conorii* binds vitronectin in part to evade serum killing. Therefore, we sought to determine if binding vitronectin would also enhance the ability of rickettsiae to adhere to and invade into target host cells such as monocytes and endothelial cells. *R. conorii* were pre-incubated with two experimentally relevant concentrations of purified multimeric and monomeric vitronectin, then exposed the bacteria to cells for a fixed time period to allow for adherence and invasion. Conversely, cells were also pre-treated with multimeric and monomeric vitronectin then added bacteria to the cells for the same time period to allow for adherence and invasion.

First, the role of vitronectin was examined in adherence and invasion of undifferentiated THP-1 cells as an *in vitro* model for circulating monocytes. When *R. conorii* were pre-incubated with 2 or 4 μg of multimeric or monomeric vitronectin, then exposed to monocytes, the ability of the bacteria to adhere to the cellular surface was not significantly affected as compared to the vitronectin-free control (Figure 3.3B). However, the ability of the bacteria to invade the cells was increased in the presence of 4 μg of multimeric and monomeric vitronectin (Figure 3.3C). Similarly, when monocytes were pre-incubated with vitronectin then exposed to *R. conorii*, there was no significant change in the ability of the bacteria to adhere to the cell surface compared to the control (Figure 3.4B). There was however, a significant increase in invasion of the bacteria in the presence of 2 and 4 μg of monomeric vitronectin (Figure 3.4C). Taken together this data demonstrates that while exogenous monomeric and multimeric vitronectin helps to facilitate the entry of *R. conorii* into monocyte-like cells, the addition of monomeric and/or multimeric vitronectin does not play a significant role in adherence.

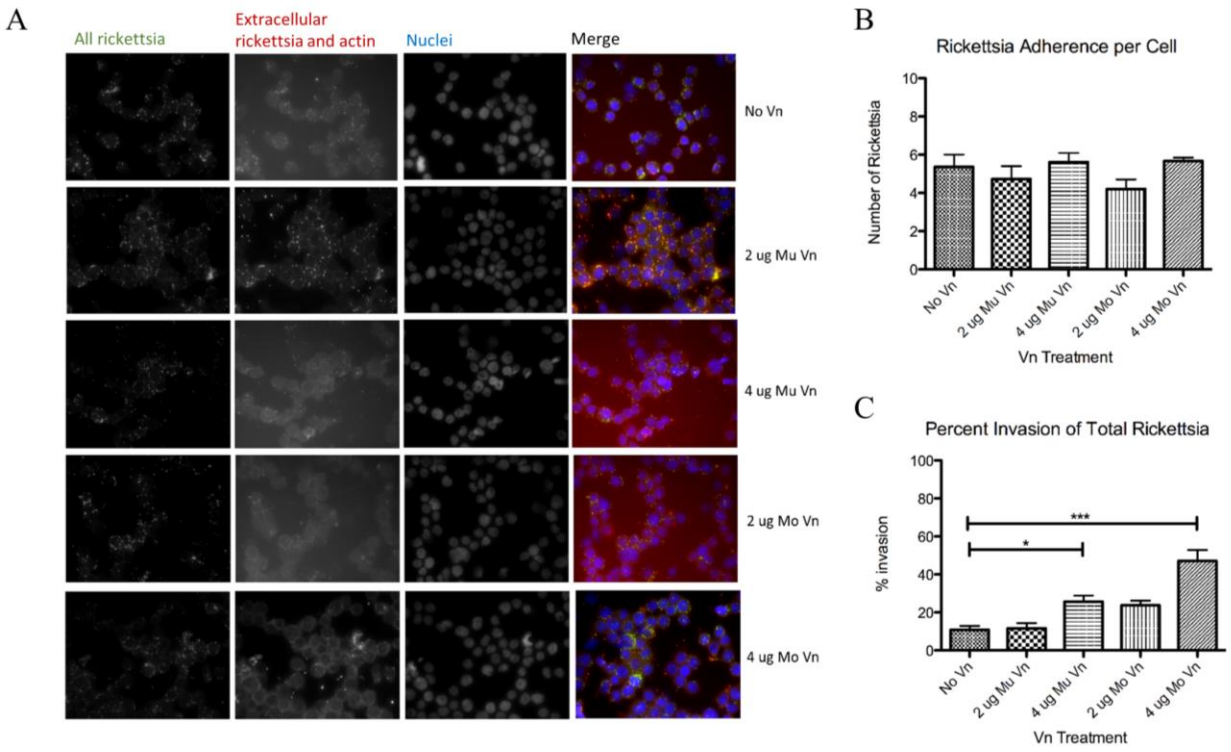

Figure 3.3. Adherence and invasion of *R. conorii* pre-incubated with vitronectin then exposed to undifferentiated THP1 cells. A. Representative immunofluorescence images of bacterial adherence and invasion assays in undifferentiated THP1 cells. Each row demonstrates, from left to right, all *rickettsia* staining, extracellular *rickettsia* and actin staining, nuclei staining and the merged image. B. Graphical representation of the average number of *rickettsia* associated with a single cell as determined by counting a minimum of 200 nuclei for each experimental condition. C. Graphical representation of the percent invasion of total rickettsia associated with a cell as determined by counting at least 200 nuclei per experimental condition. A one-way ANOVA with a Dunnet's post hoc test comparing all experimental units to a single control (no vn) was performed. * represents a $p \leq 0.05$ and is considered significant. Mo- monomeric; Mu- multimeric; Vn- vitronectin.

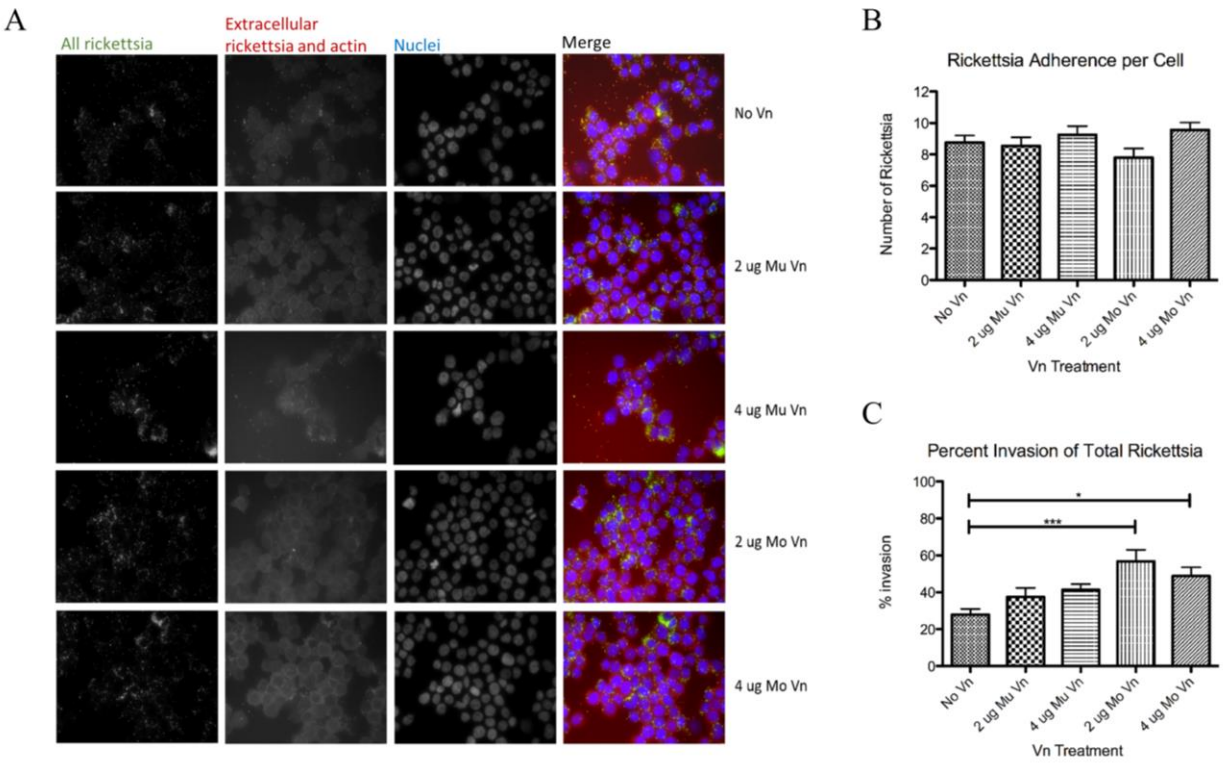

Figure 3.4. Adherence and invasion of *R. conorii* exposed to undifferentiated THP1 cells pre-incubated with vitronectin. A. Representative immunofluorescence images of bacterial adherence and invasion assays in undifferentiated THP1 cells. Each row demonstrates, from left to right, all *rickettsia* staining, extracellular *rickettsia* and actin staining, nuclei staining and the merged image. B. Graphical representation of the average number of *rickettsia* associated with a single cell as determined by counting a minimum of 200 nuclei for each experimental condition. C. Graphical representation of the percent invasion of total *rickettsia* associated with a cell as determined by counting at least 200 nuclei per experimental condition. A one-way ANOVA with a Dunnet's post hoc test comparing all experimental units to a single control (no vn) was performed. * represents a $p \leq 0.05$ and is considered significant. Mo- monomeric; Mu- multimeric; Vn- vitronectin.

Endothelial cells have long been described as a primary target of rickettsial infection and as such it is also important to examine the role vitronectin plays in adherence and invasion of this cell type (Walker and Ismail, 2008). When *R. conorii* were pre-incubated with 2 or 4 μg of multimeric or monomeric vitronectin, then exposed to EA.hy926 endothelial cells, the ability of the bacteria to adhere to the surface of the cells increased in the presence of 4 μg monomeric vitronectin compared to the vitronectin-free control (Figure 3.5B). However, this increase in adherence did not correlate with an increase in invasion. Instead, the ability of the bacteria to invade endothelial cells decreased in the presence of 4 μg multimeric vitronectin and 2 μg monomeric vitronectin (Figure 3.5C). Alternatively, when endothelial cells were pre-treated with vitronectin then exposed to *R. conorii*, the ability of the bacteria to adhere to the cellular surface was not significantly affected compared to the control (Figure 3.6B). Still, a decrease in the ability of the bacteria to invade in the presence of 4 μg monomeric vitronectin was observed. Taken together, this data suggest that *R. conorii* are better able to adhere to the surface of endothelial cells when the bacteria bind a high concentration of monomeric vitronectin prior to interaction with the cell. Surprisingly, the observed increase in adherence does not correlate with an increase in invasion. Instead, the presence of vitronectin appears to decrease the ability of the bacteria to invade endothelial cells.

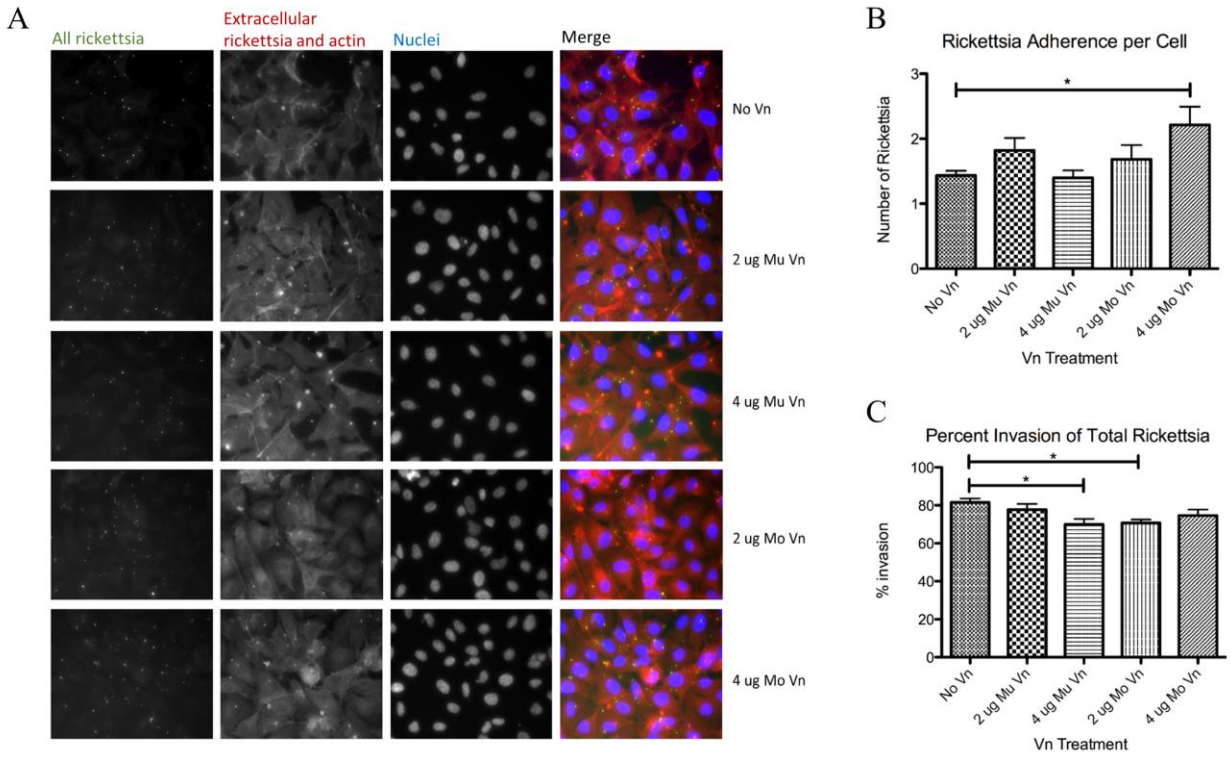

Figure 3.5. Adherence and invasion of *R. conorii* pre-incubated with vitronectin then exposed to EA.hy926 endothelial cells. A. Representative immunofluorescence images of bacterial adherence and invasion assays in EA.hy926 endothelial cells. Each row demonstrates, from left to right, all *rickettsia* staining, extracellular *rickettsia* and actin staining, nuclei staining and the merged image. B. Graphical representation of the average number of *rickettsia* associated with a single cell as determined by counting a minimum of 200 nuclei for each experimental condition. C. Graphical representation of the percent invasion of total *rickettsia* associated with a cell as determined by counting at least 200 nuclei per experimental condition. A one-way ANOVA with a Dunnet's post hoc test comparing all experimental units to a single control (no vn) was performed. * represents a $p \leq 0.05$ and is considered significant. Mo- monomeric; Mu- multimeric; Vn- vitronectin.

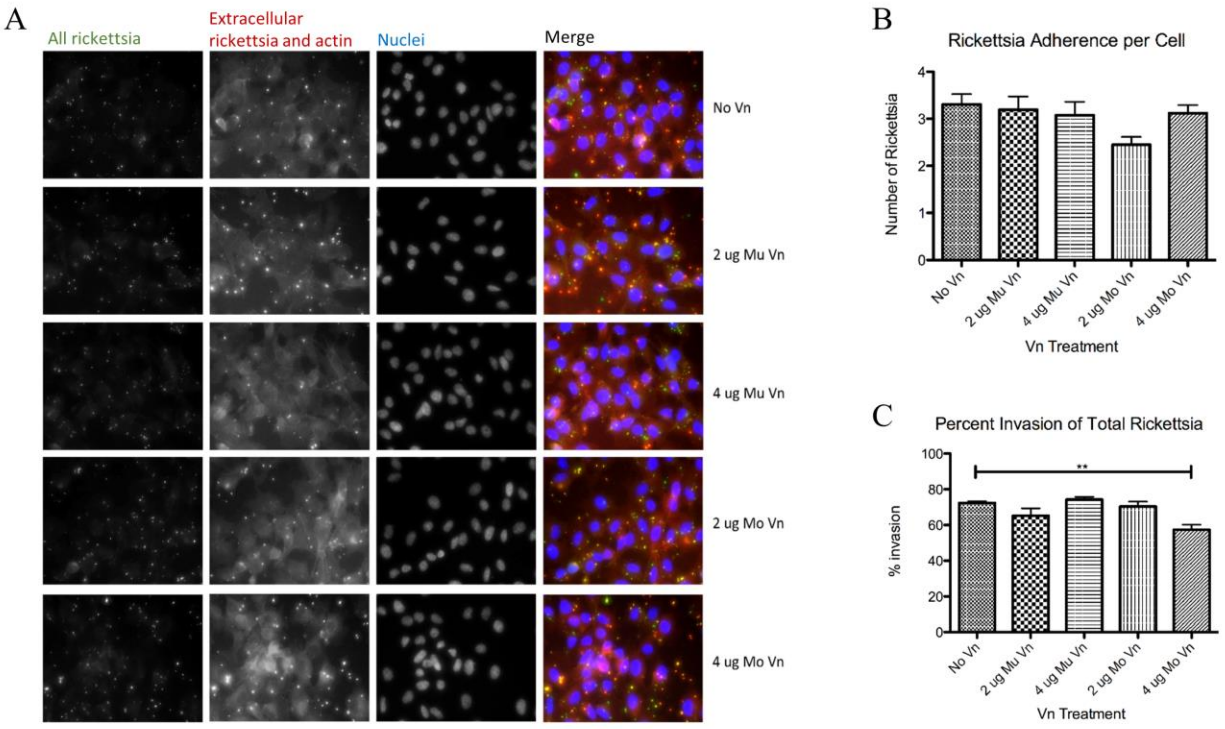

Figure 3.6. Adherence and invasion of *R. conorii* exposed to EA.hy926 endothelial cells pre-incubated with vitronectin. A. Representative immunofluorescence images of bacterial adherence and invasion assays in undifferentiated THP1 cells. Each row demonstrates, from left to right, all *rickettsia* staining, extracellular *rickettsia* and actin staining, nuclei staining and the merged image. B. Graphical representation of the average number of *rickettsia* associated with a single cell as determined by counting a minimum of 200 nuclei for each experimental condition. C. Graphical representation of the percent invasion of total *rickettsia* associated with a cell as determined by counting at least 200 nuclei per experimental condition. A one-way ANOVA with a Dunnet's post hoc test comparing all experimental units to a single control (no vn) was performed. * represents a $p \leq 0.05$ and is considered significant. Mo- monomeric; Mu- multimeric; Vn- vitronectin.

3.3.3 The $\alpha_V\beta_3$ integrin is located on the surface of endothelial cells and macrophages, but not monocytes

Differences in the ability of exogenous human vitronectin to adhere to the surface of monocytes, macrophages and endothelial cells (Figure 3.2A) suggested that the distribution of a vitronectin receptor may not be uniform among these cells types. Therefore, the distribution of the primary vitronectin receptor, the $\alpha_V\beta_3$ integrin, on the surface of undifferentiated THP-1 monocytes and EA.hy926 endothelial cells was examined. Previously, a significant increase in vitronectin-mediated invasion of undifferentiated THP-1 cells was observed suggesting that these cells likely express more $\alpha_V\beta_3$ protein at the plasma membrane in comparison to EA.hy926 cells (endothelial cells). To test this hypothesis, monocytes and endothelial cells were fixed in paraformaldehyde, stained with an antibody directed against $\alpha_V\beta_3$ and a fluorophore-tagged secondary antibody and then further processed for flow cytometry analysis. As shown in figure 3.7A, monocytes did not demonstrate a shift in fluorescence intensity compared to cells labeled with an isotype matched control antibody or secondary antibody only used as controls. In contrast, this analysis revealed that endothelial cells express this receptor at the plasma membrane as evidenced by a shift in the fluorescence intensity (red trace; Figures 3.7B and 3.7C) when compared to the controls. Taken together, the data suggests that undifferentiated THP-1 cells that model circulating monocytes do not express the $\alpha_V\beta_3$ integrin on the cell surface, while the integrin is highly expressed in EA.hy926 endothelial cells.

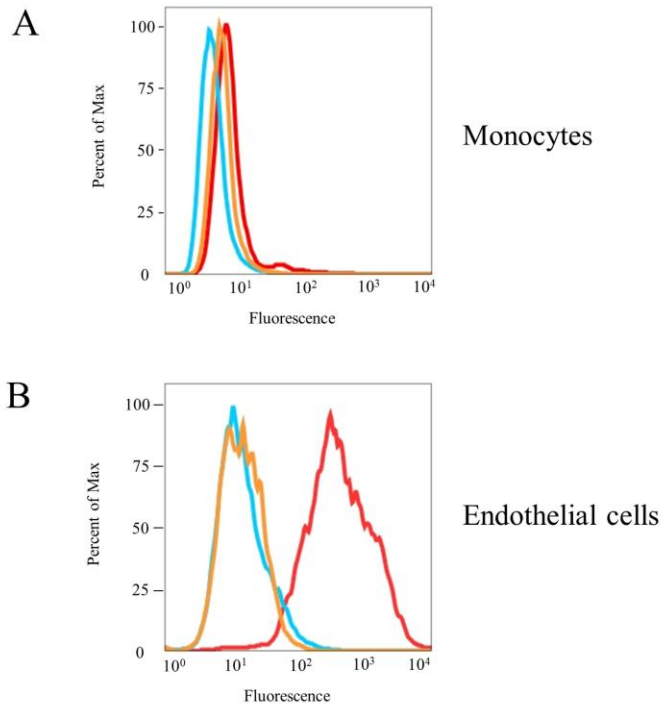

Figure 3.7. Flow Cytometric analysis of $\alpha_V\beta_3$ integrin expression on the surface of monocytes and endothelial cells. Flow cytometry demonstrated integrin expression on the surface of endothelial cells (B) by a shift in the red trace compared to cells incubated in secondary only (orange trace) or an isotype control antibody (blue trace). No integrin expression was detected on the surface of monocytes (A) as depicted by the lack of shift in the red trace.

3.4 Discussion

A previous report from the Martinez laboratory demonstrated that *R. conorii* binds vitronectin to evade complement mediated killing (Riley et al., 2014). Additional studies elucidated that both the multimeric and monomeric forms of vitronectin bound to the surface of the bacteria, but it was the multimer that offered protection from serum killing (Fish et al., 2017). The present study focused on elucidating an additional putative role for vitronectin in rickettsial pathogenesis by examining its contribution to adherence and invasion of *R. conorii* into monocytes, macrophages and endothelial cells. Human vitronectin is a multi-functional human glycoprotein that is present in circulation and in the extracellular matrix (Preissner and Seiffert, 1998). It plays a critical role in many biological processes including cell migration, tissue repair, adhesion, angiogenesis and regulation of membrane attack complex formation (Preissner and Seiffert, 1998; Singh et al., 2010b). Vitronectin has also been implicated in the development and

migration of some malignant tumors (Heyman et al., 2010; Pirazzoli et al., 2013; Zhu et al., 2015; Hao et al., 2016). The protein is arranged in a multi-domain structure that consist of an N-terminal somatomedin B domain which binds plasminogen activator inhibitor-1 and an arginine, glycine, aspartic acid (RGD) domain that interacts with multiple integrins thus promoting attachment (Schvartz et al., 1999). Vitronectin also contains three heparin binding domains and a C-terminal region with an unknown function, both of which have been demonstrated to bind surface proteins of bacterial pathogens (Blom et al., 2009; Singh et al., 2010b). The protein can exist as a folded monomer or as a partially unfolded multimer through ionic interactions with itself; however, vitronectin in the blood circulation is predominantly found as a monomer while extravascular cell-bound vitronectin is present as a multimer (Peterson, 1998; Schvartz et al., 1999). Homologs to human vitronectin are present in many mammalian species including mice, rabbits and cows, although, human vitronectin contains several distinctive amino acid residues which may be important for bacterial and human protein/protein interactions (Leduc et al., 2009). Because of its various roles, many bacterial pathogens have evolved strategies to utilize vitronectin to their advantage during the course of infection. Both Gram-negative and Gram-positive pathogens which include *Moraxella catarrhalis*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Yersinia pestis* are capable of binding vitronectin to promote adherence and invasion of host cells and to protect against complement-mediated killing (Massari et al., 2003; Sa et al., 2010; Singh et al., 2010a; Singh et al., 2010b; Su et al., 2013; Al-Jubair et al., 2015; Bartra et al., 2015; Hallstrom et al., 2015; Ikeda et al., 2015; Paulsson et al., 2015; Su et al., 2016).

The results of the current study indicated that the presence of vitronectin did not affect the ability of *R. conorii* to adhere to monocytes based on the ability of the bacteria to adhere with

similar capabilities to the cell surface when no exogenous vitronectin was added. The fact that several other rickettsial ligands which include major surface cell antigens OmpA and OmpB, have been implicated in adherence and invasion of the bacteria to host cells further supports the conclusion that the bacteria does not rely on the *R. conorii*/vitronectin interaction to bind to monocytes (Chan et al., 2009; Hillman et al., 2013). This, however, does not rule out a role for vitronectin in adherence. Instead, these results suggest that further studies are needed to elucidate its exact contribution. Nevertheless, the importance of the *rickettsia*/vitronectin/monocyte interaction was confirmed as the bacteria are better able to invade monocytes when vitronectin is present. Because vitronectin contains separate binding sites for pathogens and host cells, it most likely functions as a bridge between bacteria and cells which can also promote invasion. It has been reported that pathogens such as *Clostridium difficile, Helicobacter pylori,* and *Escherichia coli* utilize vitronectin as a bridge to better adhere to gastrointestinal epithelial cells (Ringner et al., 1992; Shen et al., 1995; Calabi et al., 2002) In addition, extracellular matrix proteins including vitronectin have been demonstrated to be important for the attachment and invasion of the intracellular pathogens *Chlamydia trachomatis* and *Burkholderia pseudomallei* to host cell surfaces (Kihlstrom et al., 1992; Tan et al., 2017). Vitronectin acquisition not only facilitates binding of pathogens to host cells, but also to synthetic materials. *Staphylococcus epidermidis,* for example, is better able to bind to polyvinylchloride (PVC) catheters in the presence of vitronectin. Antibodies directed against vitronectin significantly reduced the ability of the bacteria to adhere to the catheter when the bacteria were pre-incubated with vitronectin (Lundberg et al., 1997). Vitronectin-mediated invasion is also a well-documented characteristic for some pathogenic bacteria. *Neisseria gonorrhea, Staphylococcus aureus* and *Streptococcus pneumoniae* can better invade epithelial cells in the presence of this extracellular protein (Chhatwal et al., 1987;

Gomez-Duarte et al., 1997; Dehio et al., 1998; Bergmann et al., 2009). Interestingly, majority of the interactions that mediate invasion that have been characterized thus far involve entry into non-professional phagocytes. To my knowledge, this is the first demonstration of vitronectin mediated invasion of a professional phagocytic cell line.

Similar to monocytes, addition of vitronectin did not have an impact on the ability of *R. conorii* to adhere to endothelial cells, except when the *rickettsia* was pre-incubated with 4 μg of monomeric vitronectin. The reason for this is unclear. Perhaps, as the bacteria are exposed to higher concentrations of monomeric vitronectin, the interaction increases bacterial adherence to this cell type in a similar manner. In a previous study, it was demonstrated that an outer-membrane protein in *R. conorii* termed Adr1, was able to sequester both forms of vitronectin (Fish et al., 2017). However, resistance to serum-mediated killing was correlated with the ability of *R. conorii* to associate with the multimeric and not the monomeric form of vitronectin. In contrast to results using a model of circulating monocytic cells, invasion of *R. conorii* into endothelial cells decreased in the presence of multimeric and monomeric vitronectin. To my knowledge these results are unprecedented as there are currently no documented cases of a decrease in vitronectin-mediated bacterial invasion in the published scientific literature and most published studies have outlined the role(s) of vitronectin using epithelial cell lines. Nevertheless, *Neisseria meningitidis* is the only pathogen that has been demonstrated to employ vitronectin for an increase in invasion into endothelial cells. Specifically, an outer-membrane protein termed Opc, binds tyrosine residues exposed in the active form of vitronectin to adhere and invade human brain microvascular endothelium (Sa et al., 2010). In addition, the use of the EA.hy926 cell line which is originally derived from human umbilical vein endothelium has been utilized in the *Rickettsia* community, but may not effectively model all *Rickettsia*-endothelial cell interactions observed *in vivo* (Edgell

et al., 1983; Cardwell and Martinez, 2009; Hillman et al., 2013; Yang et al., 2016). Interestingly, cells derived from large and small vessel endothelium have differences in the expression of cell surface integrins and the patterns of expression by cultured cells are not always identical to those expressed in tissues (Albelda and Buck, 1990). It is possible that vitronectin does not play a role in the interaction of rickettsial species with endothelial cells derived from larger vessels such as the umbilical vein. As severe pathology of rickettsial infection is generally associated with infection of the microvascular endothelium, whether vitronectin plays a role in the interaction of rickettsiae with endothelial cells derived from different sources is an area that warrants further investigation.

Adherence and invasion by vitronectin bound bacteria initiates when vitronectin interacts with the host cell surface via members of the integrin family (Preissner and Jenne, 1991). Integrins are usually clustered at cell attachment sites called focal adhesion sites and mediate attachment of adherent cells through an internal signaling cascade and reorganization of the intracellular cytoskeleton network (Albelda and Buck, 1990). Engagement with an integrin by a bacteria/vitronectin complex stimulates intracellular signaling molecules resulting in actin cytoskeletal rearrangement and can trigger bacterial uptake via a "zippering" of the host cell cytoplasm around the bacteria (Singh et al., 2010b). Because it had been previously demonstrated that vitronectin interacts with monocytes and endothelial cells, surface expression of the primary vitronectin receptor, $\alpha_V\beta_3$, in these two cell types was examined. Surprisingly, the results indicated that there exists little to no surface expression of this major vitronectin receptor on undifferentiated THP-1 cells (monocytes) while the endothelial cell line, EA.hy926, expresses a large amount of the $\alpha_V\beta_3$ integrin. This suggests that the $\alpha_V\beta_3$ integrin does not facilitate adherence of vitronectin to monocytes; however, it may play a role in vitronectin adherence to endothelial cells. Although

the $\alpha_V\beta_3$ integrin has been described as the major vitronectin ligand, there are several other integrins that have been demonstrated to associate with this protein including $\alpha_3\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_5$ and $\alpha IIb\beta_3$ (Preissner and Jenne, 1991; Wei et al., 2001; Sa et al., 2010). Furthermore, both *Pseudomonas aeruginosa* and *P. fluorscens* have been demonstrated to adhere to A549 human lung epithelial cells via the $\alpha_V\beta_5$ integrin (Leroy-Dudal et al., 2004; Buommino et al., 2014). The identification of vitronectin ligands on the surface of monocytes that serve as receptors for vitronectin warrants further investigation.

Together, the results suggest that although the *R. conorii*/vitronectin interaction is dispensable for adherence of the bacteria to circulating monocytes and endothelial cells, it may in fact be a newly described mechanism by which the bacteria can attach to host cell. Also, the results of this study demonstrates a secondary role for vitronectin acquisition by the bacteria in invasion of monocytes. Although this work propagates many more questions, it is important because it contributes to our understanding of the molecular mechanisms by which pathogenic *rickettsia* species establish an infection in a mammalian host. Future directions of this work will hopefully elucidate new and successful treatments of rickettsial infection, with the ultimate goal of developing a preventative treatment.

3.5 References

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CHAPTER 4
DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

4.1 Discussion of Results and Future Directions

Because of the obligate intracellular nature of SFG *Rickettsia*, an intimate relationship with the mammalian host is necessary for the establishment of a successful infection. Interaction with the host begins immediately upon inoculation of the bacterium into the mammalian host and continues throughout its lifecycle. There are two important aspects to the initiation of infection: evasion of the host's innate immune system; and adherence and invasion of target host cells. Failure results in destruction of the bacterium by the host and prevention of infection. As a result, bacteria of the genus *Rickettsia* have evolved mechanisms to interact with the host in a manner that facilitates growth and survival. The work presented here describes the contribution of an important interaction between *R. conorii* and the mammalian host that contributes to rickettsial pathogenesis.

Of primary importance when studying intracellular bacteria are the mechanisms by which the bacteria are able to adhere to and invade target host cells as a defect, in this process is predicted to prevent successful infections. To date, several proteins have been identified in SFG *Rickettsia* that are implicated in this process. In *R. conorii*, OmpA, OmpB, Sca1 and Sca2 have been demonstrated to mediate attachment to non-phagocytic mammalian cells *in vitro*. OmpA, OmpB, and Sca2 have also been confirmed to mediate internalization of the bacterium into epithelial and endothelial cells (Cardwell and Martinez, 2009; Chan et al., 2009; Riley et al., 2010; Hillman et al., 2013). Interestingly, the predicted structures of these four proteins share homology with a family of proteins present in Gram-negative bacteria termed autotransporters (Henderson and Nataro, 2001). Additionally, autotransporters from several important human pathogens including

Haemophilus ducreyi, *Bordetella pertussis* and *Neisseria meningitides*, play critical roles as virulence determinants and have also been studied as potential targets for preventative vaccine therapies (de Gouw et al., 2014; Fusco et al., 2015; Seib et al., 2015; Gandhi et al., 2016). In addition, the causative agent of Scrub Typhus and genetically related to SFG rickettsial species, *Orientia tsutsugamushi*, expresses an autotransporter at the bacterial surface, termed ScaA, that has been demonstrated to provide protection from homologous and heterologous strains in a mouse model of infection (Ha et al., 2015).

In SFG *Rickettsia*, both OmpA and OmpB have been examined for potential use as a vaccine candidate. In a study examining the use of OmpA of *R. rickettsii* as a protective antigen, mice immunized with either an OmpA-expressing *Mycobacterium vaccae* or a DNA vaccine followed by a booster with homologous recombinant protein were protected from a lethal dose of *R. conorii* (Crocquet-Valdes et al., 2001). Additionally, Chan et al. (2001) experimented with the use of purified recombinant *R. conorii* OmpB passenger domain as a potential protective antigen. The results indicated that immunization of mice with folded OmpB, but not linear, denatured peptides generated a protective immune response against a lethal challenge with *R. conorii* (Chan et al., 2011). Interestingly, they also demonstrated that the bacteria were inherently resistant to complement mediated killing in the absence of neutralizing antibodies (Chan et al., 2011). This was the first evidence of complement resistance for this class of bacteria leading to two questions: what are the rickettsial factors involved in serum resistance; and, what are their interacting mammalian ligands? Using *R. conorii* as a model organism, Riley et al. (2012, 2014) demonstrated that the bacteria could sequester two complement regulatory proteins, factor H and vitronectin from serum, in order to subvert the host's complement system. The bacterial ligands

for factor H and vitronectin were identified as OmpB, Adr1 and Adr2, respectively (Riley et al., 2012; Riley et al., 2014; Garza et al., 2017).

Adr1 is a surface protein in *R. conorii* that is structurally related to a family of Gram-negative outer-membrane proteins exemplified by *E. coli* OmpX (Vogt and Schulz, 1999). Members of this protein family consist of eight transmembrane β-sheets which form a pore-like structure through the bacterial membrane connected by four extracellular peptides loops (Jacob-Dubuisson et al., 2004). Interestingly, homologs to Adr1 are present in every sequenced rickettsial species to date and share up to 96% sequence identify in SFG species (Riley et al., 2014). Adr1 was first identified as a protein that associates with an unknown mammalian protein and as a result was termed an adhesin (Renesto et al., 2006). Additional studies demonstrated that when expressed at the surface of a serum sensitive strain of *E. coli*, Adr1 was sufficient to confer serum resistance to the bacteria and sequester vitronectin (Vn) from human serum (Riley et al., 2014). Furthermore, expression of *R. rickettsii* (RR7045), *R. typhi* (RT815), and *R. prowazekii* (RP827) Adr1 homologs in *E. coli* also conferred serum resistance and vitronectin acquisition to levels similar to that found with *R. conorii* (Riley et al., 2014). These findings were important as they indicated that like other intracellular and extracellular blood-borne pathogens, rickettsial species bind to fluid phase complement regulatory proteins for protection from complement-mediated killing (Chhatwal et al., 1987; Bartra et al., 2008; Ho et al., 2010; Al-Jubair et al., 2015; da Silva et al., 2015; Hallstrom et al., 2015; Hill et al., 2015). Thus, the Adr1-vitronectin interaction represents another newly described pathogen-host interaction that may be important in pathogenesis.

Biochemical analysis of the Adr1/vitronectin interface demonstrated that this protein/protein interface is a heparin-independent, electrostatic interaction that is mediated by the

C-terminal region of vitronectin (Fish et al., 2017). Although electrostatic interactions have previously been established between other pathogenic bacteria and vitronectin, the Adr1-Vn interaction was unique because it was the first documented case of a heparin-independent, salt-sensitive interaction in Gram-negative bacteria. Conversely, heparin dependent, salt-sensitive interactions are well documented in the literature for both Gram-negative and Gram-positive pathogens and include proteins such as Ubiquitous surface protein A2 (UspA2) of *Moraxella catarrhalis*, Pneumococcal surface protein C (PspC) of *Streptococcus pneumoniae* and Protein E (PE) and Protein F (PF) of non-typeable *Haemophilus influenzae* (Singh et al., 2011; Su et al., 2013a; Su et al., 2013b). An additional heparin independent interaction has been identified between vitronectin and Meningococcal surface fibrils (Msf) of *Neisseria meningitides*; however, the dependence of electrostatic interactions has yet to be elucidated (Griffiths et al., 2011). Additional analysis also revealed that the Adr1-binding region of vitronectin was located in the C-terminal region adjacent to, but not including the third heparin binding domain (Fish et al., 2017). However, unlike rickettsial pathogens, the majority of other bacteria have heparin-dependent interactions and, as a result, interact with the C-terminal heparin binding domain (Singh et al., 2010; Singh et al., 2011; Voss et al., 2013; Hallstrom et al., 2016). Although the Adr1/vitronectin interaction is similar in some aspects, it is different than other bacterial/vitronectin interactions and is likely unique to rickettsial species.

In mutagenesis studies where single loop domains of Adr1 were expressed in a serum sensitive strain of *E. coli*, Riley et al. (2014) demonstrated that “loops 3” (amino acids 160-186) and “loop 4” (amino acids 210-228) provided serum resistance for the bacteria, suggesting that these loops mediate an interaction with vitronectin. Interestingly, these two peptide loops contain a high concentration of positively charged polar lysine residues and it was hypothesized that the

charged nature of these loops facilitates the interaction between Adr1 and vitronectin. Initially, single lysines within each loop were substituted for an uncharged non-polar alanine residue and the resulting protein was expressed in a serum sensitive strain of *E. coli* then exposed to human serum. The results demonstrated that a single lysine to alanine substitution at any position within loop 3 or 4 did not significantly affect the ability of the bacteria to survive serum killing (Fish et al., 2017). This data suggested that a single lysine residue was likely not important in mediating the electrostatic interaction between Adr1 and vitronectin; rather, it was hypothesized that the interplay between the proteins was mediated by the total positive charge of the loops. Additional Adr1 mutants were constructed that had sequential lysine to alanine substitutions at the first two positions, first three positions, first four position and continued until all six lysines had been mutated in each loop. Examination of the ability of *E. coli* expressing these Adr1 mutants to evade serum killing revealed that substitution of the first two or more lysines to non-charged alanine residues resulted in sensitivity to complement mediated killing when exposed to normal human serum. This indicates that the first two lysines in loops 3 and 4 are critical in mediating resistance to serum killing; however, this initial finding did not exclude the possibility that the net charge of each loop contributes to the observed phenotype (Fish et al., 2017). To test this possibility, additional double lysine mutants were constructed at lysine position 1 and 5 or at positions 5 and 6 within the Adr1 "loop 3" and then tested for their ability to survive exposure to human serum. The results demonstrated that these double lysine mutants remained resistant to serum-mediated killing suggesting that the net total charge of the loop is not critical in mediating the interaction of Adr1 with vitronectin. Instead, the results indicate that the first two lysines in loops 3 and 4 are critical in mediating the Adr1-vitronectin interactions which correlates with the ability of these bacteria to avoid serum killing (Fish et al., 2017).

Other electrostatic interactions have been described in the literature including the PspC/vitronectin interaction of *S. pneumoniae*. Although the individual amino acids responsible have not been identified, researchers speculate that the negatively charged amino acids of the R domain mediate this interaction (Voss et al., 2013). Also, a positively charged polar arginine residue has been demonstrated to be important in the interaction of vitronectin with PE of non-typeable *H. influenzae* (Singh et al., 2011). Although structurally unrelated, PspC and PE are similar to Adr1 in that these proteins are associated with the outer-membrane and have several domains exposed to the extracellular environment. Similar to PspC and PE interaction with vitronectin, the interaction between Adr1 of *R. conorii* and vitronectin is electrostatic in nature and appears to rely on the first two positively charged lysine residues within loops 3 and 4. These lysine residues in each loop are predicted to create a critical initial interaction domain that then facilitates the stabilization of the Adr1-vitronectin interaction. Taken together, it is probable that the overall structure of Adr1 plays an important role in this protein/protein interaction. As depicted in figure 4.1, it is highly likely that the first 2 lysine residues within both loops are closely associated in the overall tertiary structure of Adr1 and that the cumulative effect of these 4 lysines together creates the critical microdomain for the Adr1-vitronectin interaction. Although the first two lysine residues in loop 3 or 4 alone are sufficient to mediate this interaction, it was speculated that the interaction between intact Adr1 and vitronectin is much stronger than the interaction between a single loop and vitronectin.

Vitronectin is a multi-functional human glycoprotein that is a part of the extracellular matrix and can be found in plasma. It exists as both an internally folded and stabilized monomer, also called the native form, and a partially unfolded, functionally active multimer which can also be referred to as the active confirmation (Preissner and Seiffert, 1998). Within human plasma,

vitronectin is predominantly found as a circulating monomer with a small quantity found in the multimeric configuration. Conversely, extravascular vitronectin is primarily multimeric and cell bound. (Stockmann et al., 1993). Interaction of vitronectin with different chaotropic agents, detergents, heat or macromolecules including the membrane attack complex, has been associated with conformational transition from the monomeric form to the reactive multimeric complex (Hogasen et al., 1992; Stockmann et al., 1993).

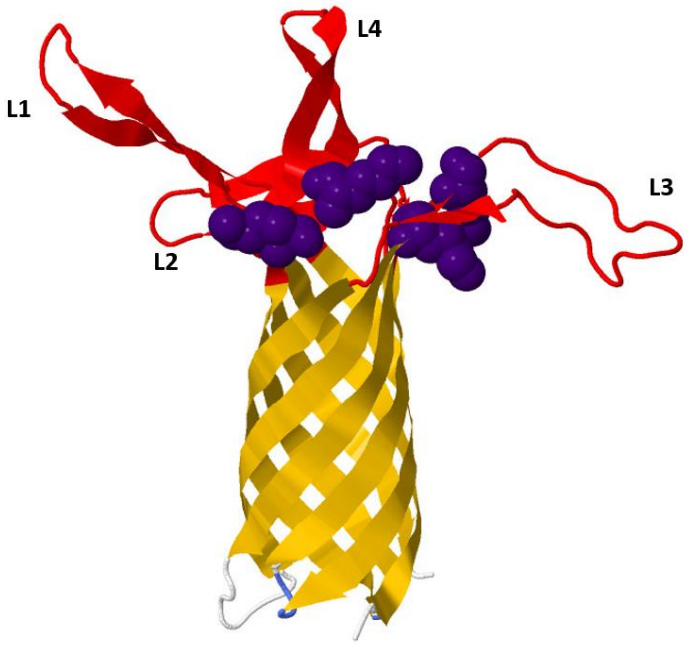

Figure 4.1. The predicted structure of *R. conorii* Adr1 with a spacefill representation of the first 2 lysine residues in loops 3 and 4. The Adr1 model demonstrates a pore-like structure with 8 transmembrane beta sheets (yellow) and 4 extracellular peptide "loop" domains (red). The purple represents a space fill model of the first 2 lysines in "loops" 3 and 4.

In the process of correlating vitronectin acquisition with serum survival of Adr1 mutants, it was discovered that Adr1 can interact with the monomeric and multimeric forms of vitronectin. However, a recent study determined that the multimer offered protection of the Adr1-expressing *E.coli* from serum killing, leaving the role of monomeric vitronectin undefined (Fish et al., 2017).

It is well-documented that both Gram-negative and Gram-positive bacterial pathogens can sequester vitronectin for evasion of serum killing and to facilitate adherence and invasion of host cells (Singh et al., 2010). These interactions are thought to be governed by the interactions of bacteria with different forms of vitronectin. For example, *Yersinia pestis* utilizes 2 outer membrane proteins Ail and Pla, to bind and process the native monomeric form of vitronectin, which is necessary to facilitate complement evasion (Bartra et al., 2008; Bartra et al., 2015). *Neisseria meningitides* preferentially interacts with the active form of vitronectin for protection from complement mediated attack and to facilitate entry into human brain microvascular endothelial cells (Dehio et al., 1998; Sa et al., 2010). Also, the respiratory pathogens, *Haemophilus influenzae* and *Streptococcus pneumoniae*, can utilize vitronectin to attach to pulmonary epithelial cells (Bergmann et al., 2009; Al-Jubair et al., 2015). Therefore, the possibility that the vitronectin/*R. conorii* interaction could also mediate attachment and invasion of target host cells was investigated.

In studies examining the role of vitronectin in adherence and invasion of monocytes and endothelial cells, addition of exogenous vitronectin did not affect the ability of the bacteria to adhere to the surface of the cell. This was not an unexpected result as previous reports have documented other rickettsial ligands including OmpA and OmpB, mediate adherence and internalization of non-phagocytic cells (Li and Walker, 1998; Cardwell and Martinez, 2009; Chan et al., 2009; Riley et al., 2010; Hillman et al., 2013). In these studies, competitive inhibition of *Rickettsia*-host cell interactions by individual purified recombinant Sca proteins was disrupted but not completely eliminated *in vitro* suggesting that other conserved factors may compensate for binding to and invasion of host cells (Cardwell and Martinez, 2009; Chan et al., 2011). In addition, the presence of vitronectin increased the ability of *rickettsia* to invade monocytes and decreased

the ability of the bacteria to invade endothelial cells. Even though this data is contradictory, these results could potentially be a result of a difference in the expression of rickettsial ligands on the surface of different mammalian cell types. Current knowledge of identified receptor/ligand pairs indicates that not all pairs mediate both adherence and internalization. Sca1 of *R. conorii*, for example, can mediate attachment of *rickettsia* to host cells, however it does not facilitate invasion (Riley et al., 2010). Because the presence of vitronectin decreases invasion into endothelial cells, it is possible that interaction of the *R. conorii*/Vn complex with the surface of EA.hy926 endothelial cells does not mediate invasion. Additionally, interaction of the bacteria with vitronectin could mask other receptor/ligand pairs that do facilitate uptake into this particular cell type. Although it is well documented that various bacterial pathogens including *Neisseria gonorrhea*, *Staphylococcus aureus*, *Burkholderia pseudomallei* and *Klebsiella pneumoniae* utilize vitronectin to enhance invasion of target host cells, to my knowledge, this is the first documented case of vitronectin reducing the ability of a pathogen to invade a cell (Chhatwal et al., 1987; Gomez-Duarte et al., 1997; Dehio et al., 1998; Tan et al., 2017). It is important to note that large and small vessel endothelium have differences in integrin expression and endothelial cells *in vitro* may not faithfully model the receptor distribution of cells in tissues (Albelda and Buck, 1990). Therefore, additional factors could potentially play a role in adherence and invasion of endothelial cells by *rickettsia* that are not effectively replicated by this *in vitro* system. Whereas most studies describing the role of vitronectin in bacterial adherence have focused on interactions with epithelial and endothelial cells, to my knowledge, this is the first description of vitronectin-mediated invasion of a professional phagocytic cell line.

Adherence to and invasion of host cells by rickettsiae that have sequestered fluid phase vitronectin is predicted to begin by binding to members of the integrin family. A plethora of

integrins have been associated with vitronectin including, the major vitronectin receptor, $\alpha_V\beta_3$, in addition to $\alpha_3\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_5$, $\alpha IIb\beta_3$ (Preissner and Jenne, 1991; Wei et al., 2001; Sa et al., 2010). When vitronectin interacts with an integrin an internal signaling cascade is triggered that results in actin cytoskeletal rearrangement and ultimately uptake of the bacteria via a "zippering" mechanism (Singh et al., 2010). Because it had previously been demonstrated that vitronectin could interact with the surface of both monocytes and endothelial cells, it was hypothesized that the major vitronectin receptor, $\alpha_V\beta_3$, would be present on the surface of both cell types. Upon examination, the $\alpha_V\beta_3$ integrin was demonstrated to be found at a high concentration on the surface of endothelial cells but was not located on the surface of monocytes. This suggests that the $\alpha_V\beta_3$ integrin may play a role in vitronectin adherence to endothelial cells, whereas another integrin likely mediates the interaction between vitronectin and monocytes. Interestingly, the bacterial pathogens *Pseudomonas aeruginosa* and *P. fluorscens* utilize the $\alpha_V\beta_5$ integrin to adhere to A549 human lung epithelial cells in a vitronectin-dependent mechanism, demonstrating that other integrins can indeed function to bind vitronectin and enhance pathogen-host cell interactions (Leroy-Dudal et al., 2004; Buommino et al., 2014). Currently, the identity of the vitronectin ligand(s) on the surface of monocytes that are utilized for rickettsial pathogens remains to be elucidated and is the subject of future lines of investigation in the Martinez laboratory.

Taken together these results demonstrate that vitronectin plays an important role in the initial stages of rickettsial infection. This body of work has demonstrated that an interaction between multimeric vitronectin and *R. conorii* Adr1 provides protection of the bacterium from complement mediated killing and the interaction between the two proteins has been characterized. Also, this work has established a role for vitronectin acquisition by *rickettsia* in invasion of the bacteria in monocytes and endothelial cells, albeit detrimental in the latter. Future work from this

study should include exploration of the detailed mechanisms of the vitronectin/bacterium interaction with both phagocytic and non-phagocytic host cells and should include exploration of the internal signaling machinery responsible for bacterial uptake. Of primary importance is the need to elucidate the host cell receptor for vitronectin on the surface of monocytes. One integrin, $\alpha_V\beta_3$, likely does not participate in this interaction; however, there are a number of other candidate proteins to investigate and the identification of the receptor would further the understanding of how pathogenic *rickettsia* species interact with phagocytic cells. The Martinez lab has previously identified two outer-membrane proteins of *R. conorii*, Adr1 and Adr2, that serve as *bona fide* vitronectin interacting proteins. It would be useful to define the contribution of Adr1 and Adr2 in vitronectin-mediated adherence and invasion of monocytes and endothelial cells. Interestingly, the predicted extracellular loop domains of both Adr1 and Adr2 are very different at the amino acid level and these differences may contribute to differences in protein function. Proposed studies would, therefore, help to determine if Adr1 and Adr2 are truly similar proteins with redundant functions or if they each have a specific role in vitronectin mediated pathogenesis of the bacteria. Interestingly, Gong et al. (2014) demonstrated that C3H/HeN mice immunized with recombinantly expressed Adr2 of *R. rickettsii* were able to survive a lethal challenge with *R. rickettsii* (Gong et al., 2014). Additionally, another human bacterial pathogen, *Neisseria meningitidis,* expresses a factor H-binding protein that has been used a protective antigen in the Meningococcal B subunit vaccine (Gorringe and Pajon, 2012; Gandhi et al., 2016). This suggests that complement binding proteins of pathogenic bacteria, including proteins such as *R. conorii* Adr1, could potentially be targets of preventative vaccine therapy.

Additionally, future studies detailing the signaling events involved in the entry of the vitronectin/*rickettsia* complex into phagocytic mammalian cells would be beneficial because it is

clear that infection of phagocytes is an important, yet under-appreciated aspect of rickettsial biology. A recent study demonstrated that the human pathogen, *R. conorii*, can survive and proliferate within the cytoplasm of differentiated THP-1 macrophage-like cells. Conversely, a non-virulent member of the SFG *Rickettsia*, *R. montanensis*, demonstrated a severe deficient in growth and survival in this cell type (Curto et al., 2016). This suggests that differences in the ability of *rickettsia* species to survive within the cytoplasm of phagocytic cells may explain the differences in pathogenicity. Furthermore, the current body of work indicates that there are at least two mechanisms by which pathogenic rickettsia can invade undifferentiated THP1 monocytes. There is a vitronectin dependent and a vitronectin independent pathway as indicated by the ability of *R. conorii* to successfully invade monocytes when no vitronectin was present. With emerging evidence to support the role of monocytes and macrophages as important cellular targets of infection, it will also be necessary to explore the detailed mechanisms by which the bacteria adhere and invade this cell type.

This work has provided a further understanding at the molecular level of how an obligate intracellular bacterial pathogen usurps host protein function for its own survival, growth and subsequent spread. In addition, this work provides further insight regarding how an organism with a small genome (1.2Mb) has evolved to take full advantage of its limited amount of resources, by expressing multi-functional proteins. Overall, the data here contributes to our understanding of the molecular mechanisms by which pathogenic *Rickettsia* species evade killing and establish an infection in a mammalian host but has also generated many more questions regarding the mechanisms by which rickettsial species and potentially other obligate intracellular bacteria initiate successful colonization of hosts. Future work in this area will hopefully enable the

development of novel therapeutic treatments for pathogenic rickettsial infections with the ultimate goal of developing a preventative treatment.

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APPENDIX A. TABLE OF PRIMER SEQUENCES USED IN MUTAGENESIS

Primer Sequences

Corresponding plasmid name	Individual Loop 3 mutants	Sequence
AF1	K160A- Forward	5'-GCTTTTGATGCGGAAAAAATTCACTCTAAAGATATTAAAGGTG-3'
	K160A- Reverse	5'-AATTTTTTCCGCTACAAAAGCAGCACCAAC-3'
	K160A- Screening	5'-GTTGGTGCTGCTTTTGTAGCG-3'
AF2	K162A- Forward	5'-GTAAAGGAAGCGATTCACTCTAAAGATATTAAAGGTGG-3'
	K162A- Reverse	5'-AGAGTTAATCGCTTCCTTTACAAAAGCAGCACC-3'
	K162A- Screening	5'-GCTGCTTTTGTAAAGGAAGCG-3'
AF3	K166A- Forward	5'-ATTCACTCTGCGGATATTAAAGGTGGTGTAACTGAC-3'
	K166A- Reverse	5'-TTTAATATCCGCAGAGTGAATTTTTTCCTTTACAAAAGC-3'
	K166A- Screening	5'-AAGGAAAAAATTCACTCTGCG-3'
AF4	K169A- Forward	5'-AAAGATATTGCGGGTGGTGTAACTGACACTTTC-3'
	K169A- Reverse	5'-TACACCACCCGCAATATCTTTAGAGTGAATTTTTTCCTTTAC-3'
	K169A- Screening	5'-GAAAAAATTCACTCTAAAGATATTGCG-3'
AF5	K181A- Forward	5'-GGTACTACTGCGAACAAAACTAACTTTGCTTATCAATTATC-3'
	K181A- Reverse	5'-AGTTTTGTTCGCAGTAGTACCGTTGAAAGTGTC-3'
	K181A- Screening	5'-ACTTTCAACGGTACTACTGCG-3'
AF6	K183A- Forward	5'-ACTAAAAACGCGACTAACTTTGCTTATCAATTATCTTTAGG-3'
	K183A- Reverse	5'-AAAGTTAGTCGCGTTTTTAGTAGTACCGTTGAAAGTG-3'
	K183A- Screening	5'-AACGGTACTACTAAAAACGCG-3'

Individual Loop 4 mutants		
AF7	K215A- Forward	5'-GATTACGGTGCGACAAAAAATACCACCAAAACTATTAAC-3'
	K215A- Reverse	5'-GTATTTTTTGTCCGCACCGTAATCTCTCCAGCTATAAAC-3'
	K215A- Screening	5'-CTGGAGAGATTACGGTGCG-3'
AF8	K217A- Forward	5'-GGTAAAACAGCGAATACCACCAAAACTATTAACGGG-3'
	K217A- Reverse	5'-GGTGGTATTCGCTGTTTTACCGTAATCTCTCCAG-3'
	K217A- Screening	5'-GAGATTACGGTAAAACAGCG-3'
AF9	K221A- Forward	5'-AATACCACCGCGACTATTAACGGGGATAAAGTTAAATTT-3'
	K221A- Reverse	5'-GTTAATAGTGCGGGTGGTATTTTTTGTTTTACCG-3'
	K221A- Screening	5'-GTAAAACAAAAAATACCACCGCG-3'
AF10	K227A- Forward	5'-AACGGGGATGCGGTTAAATTTGGTGGAACCCATTATAAAG-3'
	K227A- Reverse	5'-AAATTTAACCGCATCCCCGTAATAGTTTTGGTG-3'
	K227A- Screening	5'-CAAAACTATTAACGGGGATGCG-3'
AF11	K229A- Forward	5'-GATAAAGTTGCGTTTGGTGGAACCCATTATAAAGGTC-3'
	K229A- Reverse	5'-TCCACCAAACGCAACTTTATCCCCGTTAATAG-3'
	K229A- Screening	5'-ATTAACGGGGATAAAGTTGCG-3'
AF52	K236A- Forward	5'-ACCCATTATGCGGGTCATAACTTAATGGCAGG-3'
	K236A- Reverse	5'-GTTATGACCCGCATAATGGGTTCCACCAAATTTAAC-3'
	K236A- Screening	5'-GGTGGAACCCATTATGCG-3'

Multiple mutants in Loop 3		
AF13	pL3M2- Forward	5'-GTAGCGGAAGCGATTCACTCTAAAGATATTAAAGGTGG-3'
	pL3M2- Reverse	5'-GAGTGAATCGCTTCCGCTACAAAAGCAGC-3'
	pL3M2- Screening	5'-GCTTTTGTAGCGGAAGCG-3'
AF14	pL3M3-Forward	5'-ATTCACTCTGCGGATATTAAAGGTGGTGTAACTGAC-3'
	pL3M3- Reverse	5'-CTTTAATATCCGCAGAGTGAATCGCTTCCGCTAC-3'
	pL3M3- Screening	5'-GCGGAAGCGATTCACTCTGCG-3'
AF15	pL3M4- Forward	5'-GCGGATATTGCGGGTGGTGTAACTGACACTTTG-3'
	pL3M4- Reverse	5'-GTTACACCACCCGCAATATCCGCAGAGTGAATCGC-3'
	pL3M4- Screening	5'-GATTCACTCTGCGGATATTGCG-3'
AF16	pL3M5- Forward	5'-GGTACTACTGCGAACAAAACTAACTTTGCTTATCAATTATC-3'
	pL3M5- Reverse	5'-AGTTTTGTTGCGAGTAGTACCGTTGAAAGTGTC-3'
	pL3M5- Screening	5'-CTTTCAACGGTACTACTGCG-3'
AF25	pL3M6- Forward	5'-ACTGCGAACGCGACTAACTTTGCTTATCAATTATCTTTAG-3'
	pL3M6- Reverse	5'-CAAAGTTAGTCGCGTTCGCAGTAGTACCGTTG-3'
	pL3M6- Screening	5'-AACGGTACTACTGCGAACGCG-3'
AF53	K181A- Forward	5'-GGTACTACTGCGAACAAAACTAACTTTGCTTATCAATTATC-3'
	K181A- Reverse	5'-AGTTTTGTTCGCAGTAGTACCGTTGAAAGTGTC-3'
	K181A- Screening	5'-ACTTTCAACGGTACTACTGCG-3'
AF55	K181,183A- Forward	5'-GGTACTACTGCGAACGCGACTAACTTTGCTTATCAATTATC-3'
	K181,183A- Reverse	5'-AGTCGCGTTCGCAGTAGTACCGTTGAAAGTGTC-3'
	K181A- Screening	5'-ACTTTCAACGGTACTACTGCG-3'

Multiple mutants in Loop 4		
AF17	pL4M2- Forward	5'-GGTGCGACAGCGAATACCACCAAAACTATTAACGGG-3'
	pL4M2- Reverse	5'-GGTGGTATTCGCTGTCGCACCGTAATCTCTC-3'
	pL4M2- Screening	5'-GATTACGGTGCGACAGCG-3'
AF24	pL4M3-Forward	5'-AATACCACCGCGACTATTAACGGGGATAAAGTTAAATTT-3'
	pL4M3- Reverse	5'-GTTAATAGTCGCGGTGGTATTCGCTGTCGC-3'
	pL4M3- Screening	5'-ACAGCGAATACCACCGCG-3'
AF26	pL4M4-Forward	5'-AACGGGGATGCGGTTAAATTTGGTGGAACCCATTATAAAG-3'
	pL4M4- Reverse	5'-AAATTTAACCGCATCCCCGTTAATAGTCGC-3'
	pL4M4- Screening	5'-GACTATTAACGGGAATGCG-3'
AF28	pL4M5- Forward	5'-GATGCGGTTGCGTTTGGTGGAACCCATTATAAAGG-3'
	pL4M5- Reverse	5'-TCCACCAAACGCAACCGCATCCCCGTTAATAG-3'
	pL4M5- Screening	5'-AACGGGGATGCGGTTGCG-3'
AF29	pL4M6- Forward	5'-ACCCATTATGCGGGTCATAACTTAATGGCAGG-3'
	pL4M6- Reverse	5'-GTTATGACCCGCATAATGGGTTCCACCAAACGC-3'
	pL4M6- Screening	5'-GGTGGAACCCATTATGCG-3'

APPENDIX B. COPYRIGHT INFORMATION

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VITA

Abigail Inez Fish is the daughter of Arthur Geoffrey Fish and Mary Carolyn Fish. She was born in Andrews, Texas but spend most of her adolescent life in Dickinson, Texas. Abbie graduated from Dickinson High School in 2004 and went on to pursue an undergraduate degree at Louisiana State University with aspirations of attending Medical School. Four and half years later she graduated with a Bachelor's of Science degree in Biological Sciences and moved home to Texas to study for the MCAT. During her time at home, Abbie pursued a certificate in both basic and intermediate Emergency Medical Technician. She also worked full time a local Mexican restaurant as a server, bartender and supervisor while pursuing additional interests. During her pursuit of becoming a certified EMT, Abbie discovered a passion for infectious disease and decided to pursue a graduate degree. In 2012, Abbie began studies for a doctoral degree in Biomedical and Veterinary Medical Sciences, joining the laboratory of Dr. Juan J. Martinez. Her research interests primary focused on bacterial pathogen interactions with the host. During the course of her studies, Abbie developed a passion for science and plans to pursue a career working for the Department of Public Health for the state of Louisiana. She plans to graduate in the spring of 2018, and she will remain in Baton Rouge with her husband Brandon Berthelot.